STUDIES ON THERAPEUTIC EFFICACY OF BOVINE LACTOFERRIN IN CCL_4 AND HIGH FAT DIET INDUCED NAFLD

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CERTIFICATE

This is to certify that **Mr. K.V.VENKATARAO (ID. No. RVD/19-06)** has satisfactorily prosecuted the course of research and that the thesis **"STUDIES ON THERAPEUTIC EFFICACY OF BOVINE LACTOFERRIN IN CCL_4 AND HIGH FAT DIET INDUCED NAFLD"** submitted is the result of original work done and is of sufficiently high standard to warrant its presentation to the examination. I also certify that the thesis or part thereof has not been previously submitted by her for a degree of any University.

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CERTIFICATE

This is to certify that the thesis entitled **"STUDIES ON THERAPEUTIC EFFICACY OF BOVINE LACTOFERRIN IN CCl_4 AND HIGH FAT DIET INDUCED NAFLD"** submitted in partial fulfillment of the requirements for the degree of **DOCTOR OF PHILOSOPHY** of **P. V. Narsimha Rao Telangana Veterinary University** is a record of *bonafide* research work carried out by **Mr. K.V.VENKATARAO (ID. No. RVD/19-06),** under our guidance and supervision. The subject of the thesis has been approved by the Student's Advisory Committee.

No part of the thesis has been submitted by the student for any other degree or diploma. The published part has been fully acknowledged. All assistance and help received during the course of investigations have been duly acknowledged by the author.

The final *Viva Voce* examination was held on 29/9/2022 and the thesis is approved by the Student Advisory Committee.

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LIST OF ABBREVIATIONS

Abbreviation		Fullform
&	:	And
%	:	Per cent
@	:	At rate of
<	:	Less than
>	:	Greater than
±	:	Plus or minus
^{0}C	:	Degree (s) centigrade / Celsius
Δ Abs	:	Change in absorbance
α	:	*Alpha*
β	:	*Beta*
µg	:	Micro gram (s)
µL	:	Micro litre (s)
µM / µ mol	:	Micromole
ANOVA	:	Analysis of variance
ALT	:	Alanine amino transferase
AST	:	Aspertate amino transferase
BLF	:	Bovine lactoferrin
b.wt.	:	Body weight
BSA	:	Bovine serum albumin
CAT	:	Catalase
CCl_4	:	Carbon tetra chloride
cm	:	Centimeter (s)
cm^3	:	Cubic centimeter
CO_2	:	Carbon dioxide
DAB	:	Diamino benzidine
DEN	:	Diethyl enitrosamine
dL	:	Decilitre (s)
^{0}C	:	Degree (s)/centigrade/Celsius
DMN	:	Dimethyl enitrosamine
DMSO	:	Dimethyl sulfoxide
DPx	:	Dibutylphthalote polystyrene xylene
DTNB	:	5, 5-dithio-bis-(2-nitrobenzoic acid)
EDTA	:	Ethane-1,2-diyldinitrilo tetra acetic acid
ELISA	:	Enzyme linked immuno sorbent assay
e NOS	:	Endothelial nitric oxide synthase
ER	:	Endoplasmic reticulum
et al	:	And associates/ co-workers
Fig	:	Figure (s)
FFA	:	Free fatty acid (s)
g/gm	:	Gram (s)
GAA	:	Glacial acetic acid
GGT	:	*gamma*-glutamyl transferase
Gpx	:	Glutathione peroxidase
GSH	:	Reduced glutathione

H & E	:	Haemotoxylin and Eosin
H^+	:	Hydrogen ion
H_2O	:	Water
H_2O_2	:	Hydrogen peroxide
HCC	:	Hepatocellular carcinoma
HCL	:	Hydrochloric acid
HDL	:	High density lipoprotein
HFD	:	High fat diet
HMG-Co-A	:	3-hydroxy-3-methylglutarylcoenzyme A
hr	:	Hour (s)
HRP	:	Horse radish peroxidase
i NOS	:	Inducible nitric oxide synthase
i.p	:	Intra peritoneal route
IAEC	:	Institutional animal ethics committee
IL	:	Interleukin
IR	:	Insulin resistence
IU/L	:	International Unit/Litre
IU	:	International unit (s)
IκB	:	Inhibitory *kappa* B
JAK	:	Janus kinase
K_2HPO_4	:	Potassium hydrogen phosphate
KCL	:	Potassium Chloride
Kg	:	Kilogram
L	:	Litre
Log P	:	Partition co-efficient
LPO	:	Lipid peroxidation
Ltd	:	Limited
M	:	Molar
MDA	:	Malondialdehyde
mg	:	Milligram(s)
min	:	Minute(s)
mL	:	Milliliter (s)
mm	:	Millimeter (s)
mM	:	Milli mole (s)
MPO	:	Myeloperoxidase
MTT	:	3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyl –tetrazoliumbromide
n	:	nano
Na_2HPO_4	:	Di sodium hydrogen phosphate
NaCl	:	Sodium chloride
NAFLD	:	Non-alcoholic fatty liver disease
NASH	:	Non-alcholic steato hepatitis
NaOH	:	Sodium hydroxide
NBF	:	Neutral formaline buffer
NC	:	Normal control
NF-Kb	:	Nuclear factor – *kappa* B
NO	:	Nitric oxide
nm	:	Nanometer (s)
O_2	:	Oxygen
Oil red O	:	Oil red orange

PBS	:	Phosphate buffer solution
Pg	:	Picogram (s)
pH	:	-Log hydrogen ion concentration
Pk_a	:	Acid dissociation constant
p.o	:	Per os/ oral
Pvt	:	Private
ROS	:	Reactive oxygen species
rpm	:	Rotation per minute
SE	:	Standard error
SDS	:	Sodium dodecyl sulphate
SIM	:	Simvastatin
SOD	:	Superoxide desmutase
SPSS	:	Statistical package for social sciences
T2DM	:	Type 2 diabetes mellitus
TBA	:	Thiobarbituric acid
TBR	:	Total bilirubin
TBARS	:	Thiobarbituric acid reactive substances
TBS	:	Tris – buffered saline
TBS-T	:	Tris – buffered saline and tween 20
TC	:	Total cholesterol
TCA	:	Trichloroacetic acid
TG	:	Triglycerides
TGF-β	:	Transforming growth factor - β
TNF-α	:	Tumor necrosis factor – α
TP	:	Total protein
U	:	Unit (s)
w/v	:	Weight/volume

DECLARATION

I, **Mr. K.V.VENKATARAO (ID. No. RVD/19-06)** hereby declare that the thesis entitled **"STUDIES ON THERAPEUTIC EFFICACY OF BOVINE LACTOFERRIN IN CCL_4 AND HIGH FAT DIET INDUCED NAFLD"** submitted to P.V. Narsimha Rao Telangana Veterinary University for the degree of **DOCTOR OF PHILOSOPHY** is a result of original research work done by me. It is further declared that the thesis or any part there of has not been submitted for any other degree or diploma.

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ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) can cause Non-alcholic steato hepatitis (NASH), liver cirrhosis and hepatocellular carcinoma, which were increasing worldwide. To reduce the incidence of NASH, liver cirrhosis and Hepatocellular carcinoma (HCC), NAFLD is targeted for the development of treatments, along with viral hepatitis and alcoholic hepatitis. Bovine lactoferrin (BLF) has antioxidant, anti-cancer, and anti-inflammatory activities. It was aimed to clarify the preventive effect of BLF on NAFLD progression as its action remains unelucidated. The present study was conducted to evaluate the therapeutic efficacy of BLF in NAFLD induced by CCl_4 and high fat diet (HFD). A total of 44, healthy male C57BL/6 mice of 6-7 weeks old were procured and acclimatized for 15 days. Mice were randomly divided into 6 groups consisting of 6 mice in group 1 and 3 where as group 2, 4, 5 and 6 had 8 animals each. For the purpose of statistical analysis data (n=6) has been taken and the experiment was carried out for 6 weeks. Group 1 was kept as normal control and fed with standard diet. Remaining groups except group 3 were fed with HFD along with CCl_4 (0.5 mg/Kg b.wt in olive oil, i.p twice in a week). To induce NAFLD, Group 2 was kept as positive control for NAFLD. Group 3 as *per se* BLF (300mg/Kg b.wt p.o.) Group 4 were treated HFD +CCl_4 (0.5mg/Kg b.wt in olive oil i.p twice in a week) + BLF (300mg/Kg b.wt p.o). Group 5 was given HFD +CCl_4 (0.5mg/Kg b.wt in olive oil i.p twice in a week) + BLF (100mg/Kg b.wt p.o). Group 6 was given HFD +CCl_4 (0.5mg/Kg b.wt in olive oil i.p twice in a week) + simvastatin (10 mg/Kg b.wt p.o) for 6 weeks.

Individual body weights of all the mice were recorded on 0^{th}, 1^{st}, 2^{nd}, 3^{rd}, 4^{th}, 5^{th} and 6^{th} week of experiment. Blood samples were collected at pre-determined time intervals and serum was separated for biochemical analysis. The liver samples were

collected at the end of the experiment for absolute liver weight, histopathological examination and for analysis of anti-oxidant parameters, pro-inflammatory and anti-inflammatory markers.

The present study revealed significant ($p<0.05$) alteration in body weights, absolute liver weights, sero-biochemical parameters, inflammatory cytokines (IL-10, TNF-α, NF-κB, and TGF-β) and antioxidant profile (nitric oxide assay, TBARS, SOD, GSH and Catalase) and histopathology of group 2 as compared to other groups. The groups 4, 5 and 6 showed significant improvement in all the parameters in comparison to disease control. The values of group 3 and group 1 were comparable for significant difference.

Histopathological studies of liver in group 2 revealed degenerative changes, loss of architecture, vacuolization, moderate to severe congestion in liver. These changes were reversed in BLF treatment groups 4 (300 mg/Kg b.wt), 5 (100 mg/Kg b.wt) and simvastatin (10mg/Kg b.wt) respectively. Similarly, immunohistochemical analysis of group 2 liver revealed an intense immunopositivity for Bcl-2, while treatment groups 4, 5 and 6 exhibited mild to very mild immunoreactivity for Bcl2. However, group 3 and group 1 showed normal architecture of sections of liver tissue and did not showed any immunoreactivity for Bcl-2.

In conclusion, BLF was found to possess the ameliorating action against NAFLD induced by CCl_4 and HFD. The results showed more significant amelioration of BLF in group 4 (300 mg/kg b.wt.), which was evident in this study by reducing the pro-inflammatory cytokines and restoration of antioxidant enzymes, possibly *via* inhibiting the activation of NF-κB signalling pathway. Hence, BLF can be used as prophylactic strategy for NAFLD, with its overall beneficial effects attributed to its anti-inflammatory property. These results indicate that BLF might act as a therapeutic agent to prevent hepatic injury, inflammation, and fibrosis in NAFLD *via* NF-κB inactivation.

INTRODUCTION

CHAPTER I

INTRODUCTION

Nonalcoholic fatty liver disease has emerged as the major cause of chronic liver disease in many parts of the World (Sanyal *et al*., 2016). NAFLD constitutes a major health concern, as in most cases is intimately linked with obesity and diabetes, considering epidemic proportions in many Western countries and India. As per the official report, on 22nd February 2021, Dr. Harsh Vardhan, the Union Minister of Health & Family Welfare, GOI launched the operational guidelines for the integration of NAFLD with NPCDCS (National Programme for Prevention & Control of Cancer, Diabetes, Cardiovascular Diseases and Stroke). In the general population, the prevalence of NAFLD has been estimated at 25% - 30% and between 42% - 70% in patients affected with type 2 diabetes mellitus (T2DM) (Blachier *et al*., 2013 and Chalasani *et al*., 2018).

It has two principal clinical-histological phenotypes *i.e.* nonalcoholic fatty liver and nonalcoholic steatohepatitis (NASH). NAFLD is associated with increased cardiovascular, cancer and liver-related mortality (Wattacheril and Chalasani, 2012). Mortality is mainly due to the progression of the disease to cirrhosis (Younossi *et al*., 2016). NAFLD is characterized by evidence of hepatic steatosis, in the absence of causes for secondary hepatic fat accumulation. The presence of steatosis and inflammation with hepatocyte injury (ballooning) defines nonalcoholic steatohepatitis (NASH), which may be accompanied by progressive fibrosis (Chalasani *et al*., 2012 and Haas *et al*., 2016). It is anticipated that obesity and T2DM are the two principal risk factors for NAFLD; the burden of NASH is expected to increase over the next two decades (Ahmed *et al*., 2015).

NAFLD constitutes one of the three major causes of cirrhosis and can also be associated with occurrence of HCC (Yasui *et al*., 2011). A close relationship has been highlighted between NAFLD and the metabolic syndrome, associating visceral overweight, dyslipidemia, hyperinsulinemia or T2DM and arterial hypertension (Haas *et al*., 2016) resulting in the generally accepted conviction that NAFLD is the hepatic manifestation of the metabolic syndrome (Tsochatzis *et al*., 2009).

C57BL/6 strain in mice, Wistar and Sprague Dawley strains in rats are generally preferred because of their intrinsic predilection to develop obesity, T2DM and NAFLD (Kohli and Feldstein, 2011 and Takahashi *et al*., 2012). A previous study demonstrated that the administration of CCl_4 as an 'oxidative stress inducer' without the feeding of a HFD induced chronic oxidative stress and liver injury, but not fatty liver, steatosis or hepatocellular ballooning in mice (Kanno *et al*., 2003). Kubota *et al*. (2013) were the first to show that a combined model of HFD feeding and CCl_4 administration induced hepatic steatosis, inflammatory cell accumulation, hepatocellular ballooning, fibrosis, and increased aminotransferase levels correlating with human model.

A sequential two or multiple hit model of pathogenesis was proposed for the progression of liver steatosis to NASH; first, hepatic fat accumulation results in both macrovesicular (adipocyte accumulation) and microvesicular (hepatocyte ballooning) steatosis (Day and James, 1998 and Buzzetti *et al*., 2016). Being followed by oxidative stress in accumulated hepatic lipids (lipid peroxidation to release lipid peroxides) (Maurizio and Novo, 2005 and Sakaida and Okita, 2005) which causes inflammatory

infiltration, hepatocyte damage and fibrosis. (Sumida *et al*., 2013 and Toriniwa *et al*., 2018).

Carbon tetrachloride (CCl_4) is a well characterized liver toxin that causes direct hepatocyte injury, leading to liver fibrosis and HCC (Marques *et al*., 2012, Liedtke *et al*., 2013 and Scholten *et al*., 2015). Administration of CCl_4, in high fat diet-induced obesity (DIO) model in C57BL/6 mice resulted in histopathological features of NASH with increased serum ALT and liver hydroxyproline (Kubota *et al*., 2013). The model used a Western diet, which contained high fat, high fructose, and high cholesterol, combined with a low weekly dose of intraperitoneal carbon tetrachloride (CCl_4). This model captures the progressive stages of human fatty liver disease, from simple steatosis to inflammation, fibrosis and cancer (Tsuchida *et al*., 2018).

Statins have anti-inflammatory, anti-oxidant and anti-thrombotic effects that are independent of their lipid-lowering activity (Pignatelli *et al*., 2012 and Violi *et al*., 2013). So, they have been proposed for the treatment of NAFLD. Simvastatin was associated with amelioration of liver fibrosis by inhibition of hepatic stellate cells *via* nitric oxide synthase pathway (Wang *et al*., 2013). Nutritional supplements composed of probiotics and prebiotics are also called as symbiotics (Buss *et al*., 2014). Beneficial effects of symbiotics were also confirmed in lean NAFLD (Mofidi *et al*., 2017).

Traditionally, β -lactoglobulin, α -lactalbumin, serum albumin, immunoglobulins and proteose-peptone fractions have been considered as the major characterized components of whey proteins (Farrell and Larter, 2006; Krissansen, 2007). Lactoferrin

(LF) was first isolated from bovine milk by Sorensen and Sorensen in 1939 (Ahmed *et al*., 2021). LF exists in biological liquids such as milk, seminal fluid, and saliva (Cheng *et al*., 2008). Human milk and bovine milk, are the most plentiful source of lactoferrin.

Recently, LF has been suggested as potential preventative and adjunct treatment for Coronavirus disease (COVID-19) (Chang *et al*., 2020). It is also found in neutrophils that are released in the blood, and the inflamed infected tissues (Farnaud and Evans 2003). The pharmacological potential of LF against various pathological conditions, including oxidative stress, inflammation, fibrosis, endoplasmic reticulum stress, autophagy dysfunction, and mitochondrial dysfunction are reported.

Keeping the above facts in view, an experimental study was planned by inducing NAFLD in male C57BL/6 mice by administering CCl_4 along with high fat diet (Western diet) for a period of 6 weeks with the following objectives.

1) To evaluate the pathogenesis of CCl_4 and high fat diet in NAFLD.

2) To study hepatoprotective role of bovine lactoferrin in NAFLD induced by CCl_4 and high fat diet.

*****

REVIEW OF LITERATURE

CHAPTER II

REVIEW OF LITERATURE

NON ALCOHOLIC FATTY LIVER DISEASE

Nonalcoholic fatty liver disease (NAFLD) represents a progressive liver disorder ranging from simple liver steatosis to nonalcoholic steatohepatitis (NASH), fibrosis, cirrhosis, and ultimately hepatocellular carcinoma (HCC), in the absence of excessive alcohol intake. (Farrell and Larter, 2006, Hebbard and George, 2011). NAFLD is strongly associated with metabolic abnormalities such as obesity, insulin resistance (IR), and Type 2 diabetes (T2DM). Patients with NAFLD are at high risk of dying from cardiovascular disease and other metabolic diseases (Wanless and Lentz., 1990; Marchesini *et al*., 1999, Ma *et al*., 2017). The gold standard for confirming the NAFLD are serobiochemical and liver biopsy followed by histological examination, where hepatic triglyceride accumulation occurs in more than 5% of hepatocytes (Tandra *et al*., 2011). Macrovesicular steatosis is the predominant pattern seen in NAFLD and is characterized by large vacuoles that occupy the whole cytoplasm and push the nucleus to one side of the cell. In some NAFLD patients, multiple small lipid vacuoles are present in the cytoplasm but the nucleus remains unmoved, which is termed "microvesicular steatosis" (Lefkowitch, 2005).

Pathogenesis and Molecular Basis of NAFLD

The major factors contributing to NAFLD development are lipid accumulation in hepatocytes with inflammatory responses, cellular stress, and cell death (Cohen *et al*., 2011; Hotamisligil, 2010; Farrell *et al*., 2012 and Gual *et al*., 2017). Genetic factors and intestinal dysbiosis are also crucial (Wong *et al*., 2016). Steatosis occurs when the rate of

synthesis of lipid by hepatocytes exceeds the rate of degradation (Bradbury and Berk , 2004, Machado and Cortez-Pinto, 2014). In NAFLD intra cytoplasmic lipid accumulation with triglycerides is the most important feature in the liver pathogenesis associated with over nutrition and Insulin Resistence (Kitade *et al*., 2017, Day, 2002). Fatty acid accumulation and hyperinsulinemia causes inflammation and steatosis (Gruben *et al*., 2014; Guo, 2014, Miao *et al*., 2012). Over nutrition increases Free Fatty Acid influx from diets, resulting in de novo lipogenesis in the liver. Over nutrition also induces chronic inflammation and promotes IR (Yu *et al*., 2013, Nieto-Vazquez *et al*., 2008). Lipotoxicity is due to activation of the c-Jun N-terminal kinase (JNK) signaling pathway resulting in mitochondrial damage and hepatocyte injury (Wong *et al*., 2016, Machado and Diehl, 2016). Molecules released from damaged hepatocytes further promote changes in signaling pathways that regulate cellular stress (such as oxidative stress; endoplasmic reticulum stress) and inflammatory responses, thus perpetuating hepatocellular injury and subsequent cell death and promoting NAFLD development (Tilg *et al*., 2016). Zhang *et al*. (2014), Yu *et al*. (2006) proposed an increase in the production of proinflammatory cytokines, like tumor necrosis factor TNF- α and interleukin IL-6, and activation of toll-like receptors (TLR), activation of nuclear factor NF-κB by toll-like receptors 4 and macrophage recruitment in steatohepatitis are involved in pathophysiology of NASH (Wu *et al*., 2016). These interactions resulted in liver damage, inflammation and fibrosis, resulting in progression of NAFLD.

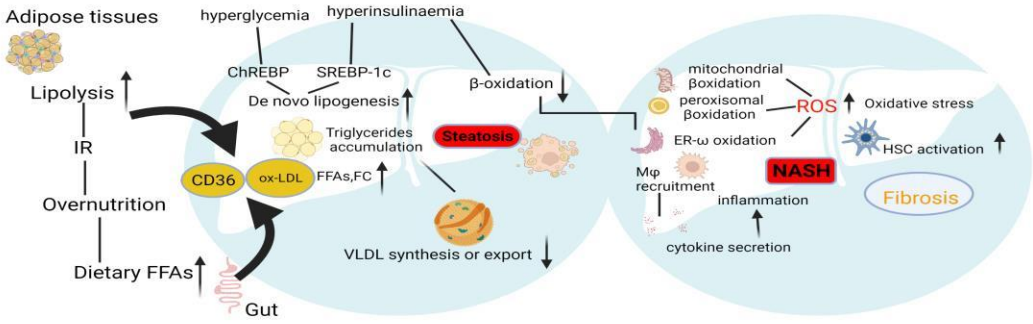

Fig. 2.1 Major processes in pathogenesis of NAFLD. (This image was created by Biorender.com).

Properties of an Ideal Animal Model of NAFLD

Main objective is to enhance the likelihood that, a drug which ameliorate NAFLD in the animal also ameliorate in humans, and then it is imperative that the model studied should mimic human disease as closely as possible. Animal models should however mimic human disease with respect to its development by diet-induced obesity, the most common risk factor for the disease in humans (Lai *et al*., 2014). Dietary composition should broadly resemble human diets in terms of their macronutrient composition and not contain unnatural toxins. Animal models should develop obesity, IR and features of systemic inflammation as seen in humans with IR (Bakiri and Wagner., 2013). These models should replicate systemic metabolic and inflammatory milieu by development of dyslipidemia and increase in inflammatory cytokines such as TNF-α, IL-6 and a decrease in adiponectin. Histologically, model should resemble human disease by having predominantly macrovesicular steatosis, lobular inflammation, hepatocellular

ballooning and hepatic fibrosis. NAFLD models not only recapitulate the diet, systemic milieu and histological spectrum of the disease but also demonstrate activation of the key cellular pathways known to be associated with human disease such as activation of de novo lipogenesis, unfolded protein response, oxidative stress, apoptosis and fibrogenic pathways (Asgharpour *et al*., 2016). Models should be reproducible and the data in the model should be repeatable. One more important signature is in terms of sensitivity to light-dark cycles, housing conditions, ambient temperature should all be further considered when selecting a specific model (Gao *et al*., 2017).

Animal Models

Animal models include dietary models like Methionine and choline deficient diet, Choline-deficient L-Aminoacid-defined diet, Atherogenic diet, Fructose & High-fat diet. Chemical models like Streptozotocin, Carbontetrachloride & Diethylnitrosamine. Genetic models like T2DM models, atherosclerosis models & HCC models. Charlton *et al*. (2011) had proposed a High fat, fructose and cholesterol diet. This diet also recapitulates features of the metabolic syndrome (e.g., Obesity; IR) and efficiently causes steatosis; hepatic steatohepatitis and fibrosis develop, but only after 24 weeks and with no evidence of pre-neoplastic nodules. Asgharpour *et al*.(2016) described a new model of NAFLD the diet-induced animal model of NAFLD, based on an isogenic strain of C57BL/J and SL29/V mice fed on HFD with *ad lib* glucose-fructose in drinking water. Oseini *et al*. (2018) used the CCl_4 model which meet most of the required criteria to be considered an ideal animal model for human NASH.

The methionine and choline deficient (MCD) diet fed model is often used as a NASH model (Phung *et al*., 2009 and Leclercq *et al*., 2000). However, feeding of an MCD diet for 4 weeks has been reported to cause macrovesicular steatosis with moderate inflammation but negligible fibrosis, (Sahai *et al*., 2004), and for 10 weeks to develop early stage fibrosis in mice, (Leclercq *et al*., 2000). Above all, long-term feeding (>6 months) of an HFD is required for the development of histological steatohepatitis, making this a time-consuming model for establishing NASH pathology (Svegliati *et al*., 2006 and Ito *et al*., 2007).

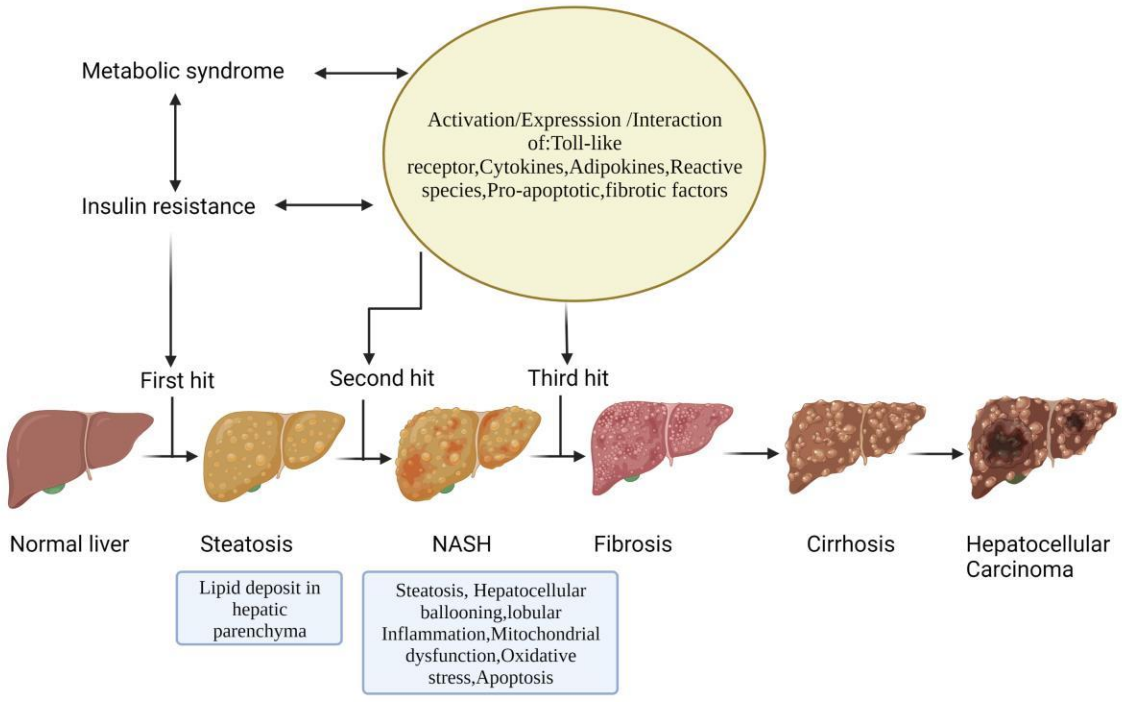

Fig. 2.2 The multi hit hypothesis and pathophysiology of NAFLD. (This image was created by Biorender.com).

High Fat Diet (HFD)

Rats fed on high fat diet composed of 71% fat, 11% carbohydrates, and 18% proteins for 3 weeks is known to develop insulin resistance with marked panlobular steatosis, inflammation and induce fibrosis (Asgharpour *et al*., 2016), whereas the mice fed with HFD showed similar results around 16 weeks. Thus, the key feature of this model is that the results vary with rodent strain and the composition of the diet (Nakamura and Terauchi, 2013). The C57BL6/J mice are more insulin resistant and thus more likely to be relevant (Haluzik *et al*., 2004). Many advances in the development of preclinical models for NAFLD have been made that have provided valuable insights on disease pathogenesis.

Established animal models have vividly highlighted the important aspects of each stage of NAFLD and provide significant clues to the critical molecular events during NAFLD development, which opens up new opportunities for treatment of NAFLD in humans. The main calorie intake (energy) of HFD is derived from fat (45% to 75%). Animals fed with HFD can replicate the major histopathology and pathogenesis seen in human NAFLD. Rats fed HFD developed steatosis, IR, mitochondrial dysfunction, and mononuclear inflammation, accompanied by increased hepatic TNF- α and CYP2E1 induction. (Lieber *et al*., 2004). Male C57BL/6 mice fed a HFD (60% fat, 20% carbohydrates, and 20% protein) for 12 weeks developed steatosis (Jiang *et al*., 2019).The model used a Western diet, which contained high fat, high fructose, and high cholesterol, combined with a low weekly dose of intraperitoneal carbon tetrachloride (CCl4). This model captures the progressive stages of human fatty liver disease, from simple steatosis to inflammation, fibrosis, and cancer (Tsuchida *et al*., 2018).

2.1.5 Effect of NAFLD on Body Weight

Chang *et al.* (2021) investigated the therapeutic effects of Saikosaponin-d (SSd) treatment and HFD induced NAFLD in male C57BL/6 mice, showed reduced body weight when compared with the HFD-fed control group to the SSd group. Gaemers *et al*. (2011) noticed that feeding C57BL/6J male mice *ad libitum* HFD resulted in a bodyweight gain, absolute weights and relative weights of liver over a period of 12 week. Xia *et al*., (2019) study on green tea polyphenols (GTP) effects against NAFLD reported that the weight of HFD fed rats gradually increased after 2 weeks and was significantly higher compared with the normal control group However, when the rats were pretreated with GTP, the weight gain was slower significantly less compared with the HFD rats.

Effect of NAFLD on Glucose

Ore, *et al*. (2020) reported that plasma glucose level in mice was high in the HFD fed group compared to control, although the increase was not significant. Gaemers *et al*. (2011) stated that plasma glucose levels did not differ significantly between chow fed and HFD fed mice in either the fasted or the non-fasted condition. Xia *et al*. (2019) study on GTP effects against NAFLD showed that fasting serum glucose and insulin resistance were decreased in the Green tea polyphenols treated rats.

Effect of NAFLD on Sero-Biochemistry

Chang *et al*. (2021) in their study, compared with the control group, the Saikosaponin-d group had lower hepatic mRNA levels of fatty acid–binding protein 4 (FABP4) and sterol regulatory element–binding protein 1(SREBP1) respectively.

Therefore, Saikosaponin-d reduces gene-associated hepatic steatosis, significantly decreasing AST and ALT levels, and could improve NAFLD symptoms, mediating the mRNA expression of molecular mechanisms to affect hepatic lipid accumulation**.** Compared with the control group, serum triglycerides significantly decreased in the Saikosaponin-d group. Ore *et al.* (2020) showed that the biomarkers of liver function like

ALT, AST, and alkaline phosphatase increased significantly in the HFD group compared with the control group. Also, reported the TC and TGs levels, increased significantly in the plasma of mice in the HFD group compared to the control group. Plasma level of HDL cholesterol decreased significantly in the HFD group compared to control. Gaemers *et al*. (2011) stated that plasma TG levels decreased with time in mice on the HFD. Plasma cholesterol levels were increased in HFD fed mice, plasma Non Esterified Fatty Acid (NEFA) levels were not significantly different at any time point between any of the diets, plasma ALAT, ASAT, AP, γ-GT or bilirubin levels were not significantly elevated in the experimental groups. Zou *et al. (*2020) found that an HFD caused a lipid metabolism disorder in mice, significantly increased the serum TC and TG levels, significantly reduced HDL levels and increased activity of AST and ALT. Yang *et al*. (2019) investigated the hepatoprotective effects of quercetin in T2DM induced NAFLD, the results revealed that quercetin reduced alleviated serum transaminase activity and restored the increased serum total bile acid, serum cholesterol, and triglycerides levels. Xia *et al*. (2019) study on GTP effects against NAFLD significantly reduced serum ALT, AST, TG, TC and NEFA in liver of Green tea polyphenols treated rats.

Effect of NAFLD on Antioxidants

Ore *et al.* (2020) observed the status of hepatic antioxidant and oxidative stress markers, where there is a significant reduction in the activity of GSH and GSH-Px in the liver of mice in the HFD group compared to CD group. Hepatic MDA content and protein carbonyls increased significantly in the HFD group compared with control. Gaemers *et al*. (2011) proposed that fraction of oxidized glutathione (GSH/GSSG) did not differ between the experimental groups. Yang *et al*. (2019) found that quercetin restored superoxide dismutase, catalase, and glutathione activity in liver. Xia *et al*. (2019) study on Green tea polyphenols (GTP) effects against NAFLD reported that the MDA was significantly increased in the liver of the HFD rats compared with the control group while the activity of SOD was notably decreased. GTP significantly decreased the level of malondialdehyde whereas superoxide dismutase level was increased in the liver.

Effect of NAFLD on Cytokines

Zou *et al.* (2020) studies have found that under NAFLD conditions, the liver develops inflammation and releases a large number of inflammatory cytokines (TNF-α, IL-1β, and IL-6) at a higher level. Chang *et al*. (2021) reported that in NAFLD mice there was an increased expression of inflammation related genes nuclear factor kappa B (*NF-κB*) and inducible nitric oxide synthase (*iNOS*)) messenger RNA (mRNA) compared with controls. Yang *et al*. (2019) suggested that quercetin markedly attenuated T2DM induced production of interleukin 1 beta, interleukin 6, and TNF- α. Santi *et al*. (2016) studied the effect of high-carbohydrate diet (HCD) and HFD modulating the liver inflammatory state,

was evaluated by the measuring the myeloperoxidase activity, NO inflammatory gene expressions like IL-6, TNF- α, NF-κB and IL-10. Ore *et al*. (2020) reported that there was a significant increase in the level of TNF- α in HFD group compared to the CD group. Gaemers *et al*. (2011) stated that plasma TNF-α levels were significantly increased at all time points in the HFLD-overfed mice only. Xia *et al*. (2019) study on GTP effects against NAFLD, the serum levels of the inflammatory cytokines TNF- α and IL- 6 were notably increased in the HFD rats compared with the control group, and GTP treatment decreased the levels of TNF- α and IL- 6 in the HFD rats suggest that GTP inhibits HFD- induced oxidative stress and the inflammatory response in the liver of rats.

Effect of NAFLD on Histopathology

Ore *et al*. (2020) noticed in the HFD group, microvesicular steatosis with hepatocellular ballooning in the zone 3 hepatocytes. Gaemers *et al*. (2011) observed that in the HFLD-overfed mice, initially a homogeneous accumulation of small lipid vesicles were observed. However, after 12 weeks of overfeeding, large lipid droplets were present around the portal veins in addition to the homogeneously distributed smaller lipid vesicles. Zou *et al. (*2020) viewed liver sections from the control group showed normal cell morphology, with well-preserved cytoplasm, a prominent nucleus, a central vein (CV), and a compact arrangement of hepatocytes. In contrast, the liver cells of the HFD group showed significant anomalies, with hydropic changes in centrilobular hepatocytes, and necrosis of the neutrophils being observed. Yang *et al*. (2019) observed in Oil red O staining significant lipid droplet accumulation under model conditions, whereas quercetin could eliminate lipid droplets in a dose- dependent manner. Liver injury in NASH

patients is associated with apoptosis and NF-κB activation even though anti-apoptotic B-cell lymphoma 2 (Bcl-2) is strongly expressed (Ribeiro *et al.*, 2004, Ramalho *et al*., 2006, Li *et al*., 2014, and Kanda *et al*.,2018).

CARBON TETRACHLORIDE

CCl_4 is widely used as a solvent for dissolving non-polar compounds, such as fats and oils. The acute toxicity of CCl_4 is well established from many animal studies. Single oral doses of CCl_4 in corn oil induce increased liver weight, elevated levels of fat, serum urea, liver enzyme activities, and clear histopathological evidence of liver damage with single cell necrosis (Korsrud *et al*., 1972). Toxicity of CCl_4 has three or four distinct phases, the first two or three weeks are mainly characterized by necrosis indicated by rising activities of liver specific enzymes and decreasing values of pseudocholinesterase. During the next two or three weeks massive hepatic fat accumulation occurs and serum levels of triglycerides and AST activity are significantly increased, where as hepatic function is reduced. During the third phase, the increase of AST continues, elevated levels of hydroxyproline and triglycerides are found, and overall liver function further decreases. In the final phase, the values of pseudocholinesterase further decrease, and atrophy of the liver is observed. (Paquet and Kamphausen, 1975).

Pathogenic Mechanisms of Liver Damage

CCl_4 is metabolized in the liver by the cytochrome P450 superfamily of monooxygenases (CYP family) to the trichloromethyl radical (CCl_3^*). This radical reacts with nucleic acids, proteins, and lipids, thereby impairing key cellular processes resulting in altered lipid metabolism (fatty degeneration and steatosis) and lowered protein. The

formation of trichloromethylperoxy radicals (CCl_3OO^*) resulting from oxygenation of CCl_3^* further initiates lipid peroxidation and the destruction of polyunsaturated fatty acids. The membrane permeability in all cellular compartments (mitochondria, endoplasmic reticulum, and plasma membrane) is lowered and generalized hepatic damage occurs that is characterized by inflammation, fibrosis, cirrhosis and HCC (Weber *et al*., 2003). C57BL/6 in-bred mice strain is frequently used for fibrosis studies which develop only intermediate liver fibrosis. (Hillebrandt *et al.*, 2002 and Walkin *et al*., 2013) proposed, CCl_4 can be administered through i/p injection, inhalation and gavage. The majority of investigators prefer i.p administration in mice for reasons of excellent reproducibility, good survival rates, (Chang *et al*., 2005). Administration of CCl_4 *via* gavage route is not to be recommended due to frequent early mortality (McLean *et al*., 1969).

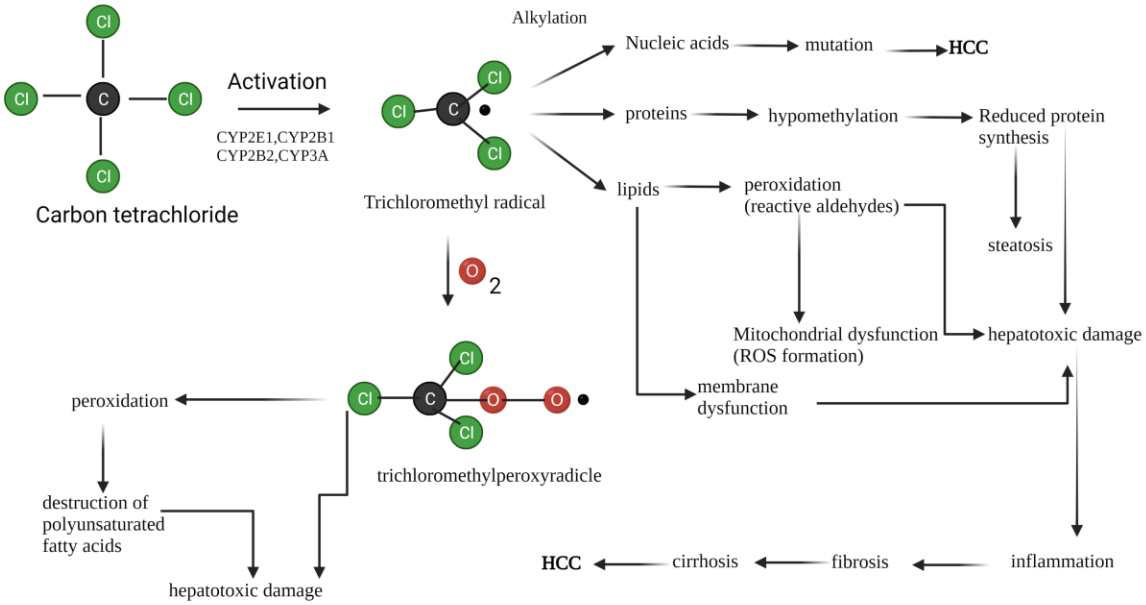

Fig. 2.3 Patho biochemical sequence of events during CCl4 inducing liver damage (This image was created by Biorender.com).

Side-Effects

Intraperitonial administration of CCl_4 lead to local irritation of the skin and can cause mild peritoneal inflammation. Long-term complications like portal hypertension, oesophageal bleeding, ascites, abdominal adhesions, hepatic inflammation and transient spasms. Animals should be re-inspected one hour after injection for abnormalities and every 24 hr thereafter. Established fibrosis can best be investigated after 2 to 4 weeks of CCl_4 treatment, while severe fibrosis (cirrhosis) can be observed after 6 to 8 weeks of treatment. CCl_4 causes liver damage through a number of mechanisms like formation of reactive free radicals that can bind covalently to cellular macromolecules forming nucleic acid, protein and lipid adducts, affecting hepatocellular

calcium homeostasis. CCl_4 causes centrilobular steatosis, inflammation, apoptosis and necrosis further then progress to fibrosis and cirrhosis (Manibusan *et al*., 2007; Weber *et al*., 2003 and Shi *et al*.,1998). Multiple reports have revealed considerable disadvantages in the intraperitoneal, subcutaneous, inhalation and oral routes of CCl_4 administration, including chronic peritonitis, necrosis at injection site with consistent fibrosis, respiratory arrest and higher mortality with inconsistent fibrosis, respectively (Starkel and Leclercq, 2011).

Effect of CCl_4 on Body Weight

Huang *et al*. (2020) proposed that the body weights of the CCl_4 treatment group mice were significantly decreased, whereas weights were gradually recovered by total phenolic compounds extracted from *E. konishii* pericarp (TPEP) treatment. Kubota *et al*. (2013) reported that CCl_4 and HFD fed group mice showed increase in bodyweight and absolute weights of liver as much as those in the normal diet group. Chheda *et al*. (2014) stated that animals on FFD with a micro dose of CCl_4 (0.5 ml/Kg b.wt, p.o) showed weight gain and absolute weights of liver when compared to the chow diet-fed animals. Owada *et al*. (2017) indicated that body weights, absolute weights and relative weights of liver were increased in the CCl_4 group.

Effect of CCl_4 on Glucose

Jaisheela *et al*. (2021) reported that the rats administrated with CCl_4 showed a greater increase in the level of glucose when compared with the control group. However, the level of glucose showed significant decrease in serum of rats that were treated with the miniaturized scaffold of silymarin and quercetin. Ishtiaq *et al*. (2022) reported high

blood glucose levels in HFD and CCl_4 treated group where as silymarin, kaempferol and rosiglitazone treatment significantly lowered the blood glucose levels. Kubota *et al.* (2013) observed CCl_4 and HFD fed group mice showed lower serum glucose levels when compared with those fed the normal diet. Chheda *et al*. (2014) did not observe any changes in blood glucose levels under both fasting and fed condition across all the groups in their study.

Effect of CCl_4 on Sero-Biochemistry

Huang *et al*. (2020) stated that activities of ALP, ALT and AST were remarkably increased in contrast with the control group suggesting liver function was severely impaired by CCl4 induction wherein the ALT, AST and ALP levels were dramatically decreased by total phenolic compounds extracted from *E. konishii* pericarp (TPEP) administration. Owada *et al.* (2017) indicated that the serum TG, ALT and AST activity were significantly increased in the HFD+CCl_4, a novel NASH model group, relative to the other groups. Further, the serum cholesterol level was significantly decreased in the novel NASH model group relative to the other groups. Chheda *et al*. (2014) expressed that ALT, AST, GGT, ALP (known serum markers of liver injury) and procollagen type III (known serum marker of fibrosis) were significantly elevated, serum triglyceride levels were reduced significantly in the FFD+CCl_4 animals compared to the chow diet controls. Tanaka *et al*. (1999) noticed that AST and ALT levels were significantly higher in CCl_4 treated rats than in controls and the plasma nitrite/nitrate concentration was significantly higher in the CCl_4 treated rats than in controls. Li *et al*.

(2018) suggested that the serum activity of ALT and AST were much higher in the model group, whereas treatment with *Periplaneta americana* extracts, altered ALT and AST activity.

Effect of CCl_4 on Antioxidants

Huang *et al*. (2020) noticed that CCl_4 administration greatly impaired the activities of CAT, GSH-Px and SOD, but increased MDA values, total phenolic compounds extracted from *E. konishii* pericarp (TPEP) treatment gradually upregulated the antioxidant enzymes activities and lessened the MDA values. Chheda *et al*. (2014) reported a significant depletion in liver glutathione in FFD+CCl_4 animals compared to FFD and chow diet controls whereas, liver TBARS were elevated in both FFD and FFD+CCl_4 animals.

Effect of CCl_4 on Cytokines

Li *et al*. (2018) observed that the expression levels of NF-κB and TGF-β were markedly higher in the CCl_4 group than those in the normal group. Huang *et al*. (2020) proposed that CCl_4 induction remarkedly elevated both the protein and mRNA levels of TNF-α, IL-6 and monocyte chemotactic protein 1 (MCP-1) in the liver which could be suppressed by total phenolic compounds extracted from *E. konishii* pericarp (TPEP) treatment, the study showed that TPEP attenuated the climbing protein and mRNA expressions of TGF-β, Smad 2 and Smad 3 induced by CCl_4 administration ,but upregulated both the protein and gene levels of Smad 7. Chheda *et al*. (2014) noted that TNF-α (proinflammatory cytokine implicated in fibrogenesis) value increased both in the FFD and FFD+CCl_4 animals, whereas TGF-β (marker of fibrosis and inflammation) was

expressed at significantly higher levels in FFD+CCl_4 animals compared to the FFD, CCl_4 and chow diet controls. Owada *et al*. (2017) reported that the serum TNF-α and IL-6 concentration levels were significantly higher in the novel NASH model group relative to the other groups.

Effect of CCl_4 on Histopathology

Huang *et al*. (2020) observed that histopathological examination, showed the normal liver has complete hepatocyte structure, clear hepatic cords architecture and almost no fibrous hyperplasia in the liver tissues and Masson's trichome staining (MTS) revealed that CCl_4 administration resulted in the connective tissue proliferation, fibrous collagen deposition and lymphocytes infiltration in the mice liver indicating severe liver fibrosis. However, the extent of hepatic steatosis and necrosis was gradually reduced, the collagen deposition and inflammatory infiltration was attenuated with the use of total phenolic compounds extracted from *E. konishii* pericarp. Kubota *et al*. (2013) reported that HFD group developed steatosis, in which hepatocytes were seen to store fat droplets, however, no recruitment of inflammatory cells or hepatocellular ballooning, but mice in the CCl_4+HFD group showed lobular inflammation together with hepatocellular ballooning, as well as apparent pericellular and perisinusoidal fibrosis in the liver. Chheda *et al*. (2014) stated that histological examination of liver sections from FFD+CCl_4 animals showed a significant increase in micro vesicular and macro vesicular steatosis, hepatocellular ballooning and fibrosis. Masson's trichome staining (MTS) and picrosirius red staining were carried out to evaluate collagen distribution, liver sections from FFD and FFD+CCl_4 animals, showed increased collagen deposition with the latter showing moderate to severe collagen deposition detectable as perisinusoidal fibrosis and

bridging fibrosis. Owada *et al*. (2017) observed that the novel NASH model group developed macrovesicular hepatic steatosis, ballooning hepatocyte with Mallory-Denk bodies, lobular inflammation and bridging fibrosis. Li *et al*. (2018) reported that MTS and H&E staining of liver tissue samples from rats in the normal group displayed normal lobular architecture with central veins, uniform cells, and neat rows of hepatic cords without degeneration and there was no evidence of fibrous tissue hyperplasia or pseudolobule and liver cell necrosis or collagen fibers. In contrast to those from the normal group, liver tissue sections from the model group displayed a markedly high number of inflammatory cell infiltrates, pronounced adipose degeneration of hepatocytes, extensive necrosis of hepatocytes around the lobule and increased deposition of collagen fibers in hepatic lobules, and the formation of a pseudo lobule that separated the lobules. But, histopathological examination of treatment groups liver sections, indicated adipose degeneration and necrosis of hepatocytes, were markedly ameliorated, and migration and infiltration of inflammatory cells were reduced in the groups treated with *Periplaneta americana* extracts.

Statins

Several medications including thiazolidindiones, metformin, vitamin E, statins, pentoxifylline, losartan, ursodeoxycholic acid, probiotics and symbiotics have been applied for treatment of NAFLD/NASH with promising but conflicting results. Statins that inhibit hydroxyl-methyl-glutaryl-coenzyme A reductase are widely used as cholesterol lowering agents and can be theoretically useful in patients with NAFLD (Mihos *et al*., 2014).

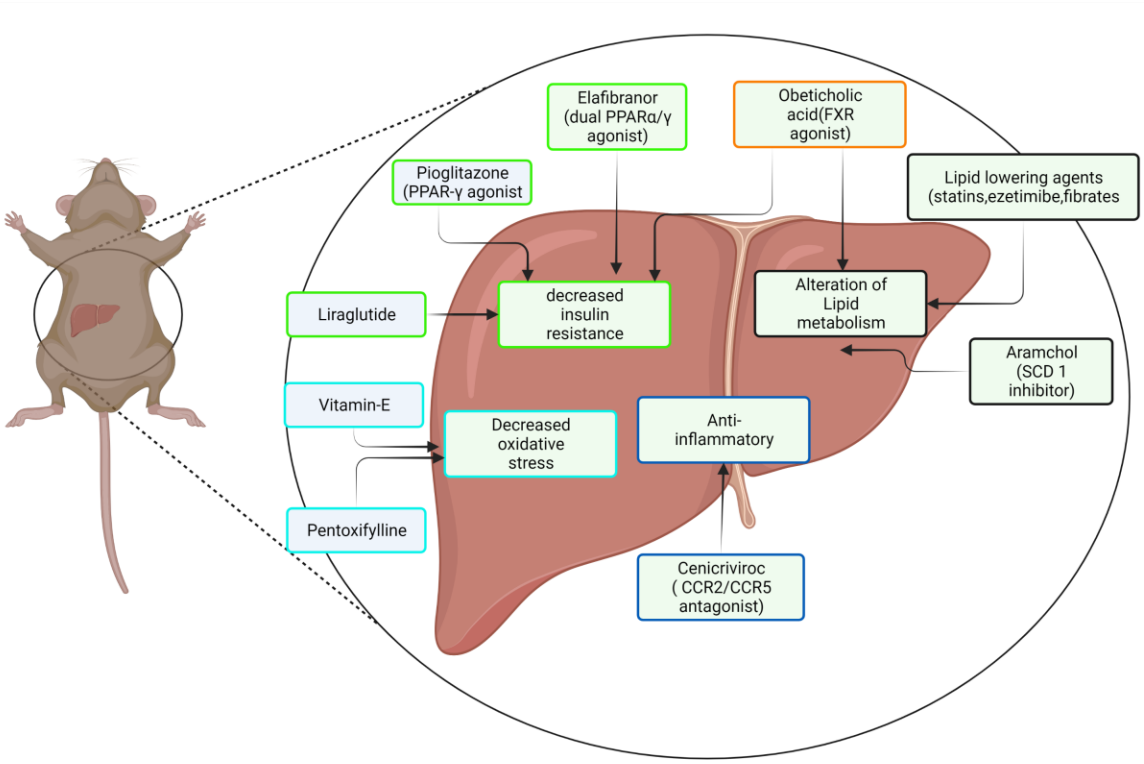

Fig. 2.4 Current and Emerging drugs for NAFLD and their mechanism of action (This image was created by Biorender.com).

Statins, inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, have revolutionized the treatment of hypercholesterolemia. They are the most efficient agents for reducing plasma cholesterol, being also appreciated for their good tolerance. Angiographic studies have demonstrated that these compounds reduce the progression and may induce the regression of atherosclerosis (Vaughan *et al.*, 2000). The beneficial effects of the HMG-CoA reductase inhibitors are usually attributed to their capacity to reduce the endogenous cholesterol synthesis, by competently inhibiting the principal enzyme involved (Hunninghake, 1992). Statins have direct action on lipids, or intracellular signaling pathways, it includes inhibition of cholesterol biosynthesis,

increased uptake and degradation of LDL, inhibition of the secretion of lipoproteins, inhibition of LDL oxidation, and inhibition of the scavenger receptors expression (Bellosta *et al.,* 2000). Statins modulate a series of processes leading to reduction of the accumulation of esterified cholesterol into macrophages, increase of eNOS, reduction of the inflammatory process, increased stability of the atherosclerotic plaques, restoration of platelet activity and of the coagulation process.

Mechanisms for the Action of Statins

Statins target hepatocytes and inhibit HMG-CoA reductase, the enzyme that converts HMG-CoA into mevalonic acid, a cholesterol precursor. The statins do more than just compete with the normal substrate in the enzyme's active site, they also alter the conformation of the enzyme when they bind to its active site. This prevents HMG-CoA reductase from attaining a functional structure. The change in conformation at the active site makes these drugs very effective and specific. Binding of statins to HMG-CoA reductase is reversible, and their affinity for the enzyme is in the nanomolar range, as compared to the natural substrate, which has micromolar affinity (Corsini, 1999).

Statins such as atorvastatin and simvastatin in clinical studies were associated with a reduction in hepatic steatosis and can inhibit the progression of NASH (Eshraghian, 2017; Bril *et al*., 2017 and Blais *et al*., 2016). Simvastatin was also reported to lower the elevated liver enzymes and reduce hepatic fatty infiltration in patients with NAFLD (Abel *et al*., 2009) and stabilize or reverse fibrosis (Ekstedt *et al*., 2007) by inhibiting HSC proliferation (Rombouts *et al*., 2003). Simvastatin is one of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors which have lipid-lowering

property. In addition, it also exhibits potential benefits such as anti-inflammation, anti-oxidation, improving endothelial dysfunction, increasing nitric oxide bioavailability, stabilizing the atherosclerotic-plaques, decreasing intrahepatic vascular resistance, and reducing portal pressure (Khan *et al*., 2009 and Zafra *et al*., 2004). Zamin *et al*., (2010) stated that simvastatin not only reduced circulating low-density lipoprotein cholesterol but decreased hepatic lipid deposition in patients with NASH.

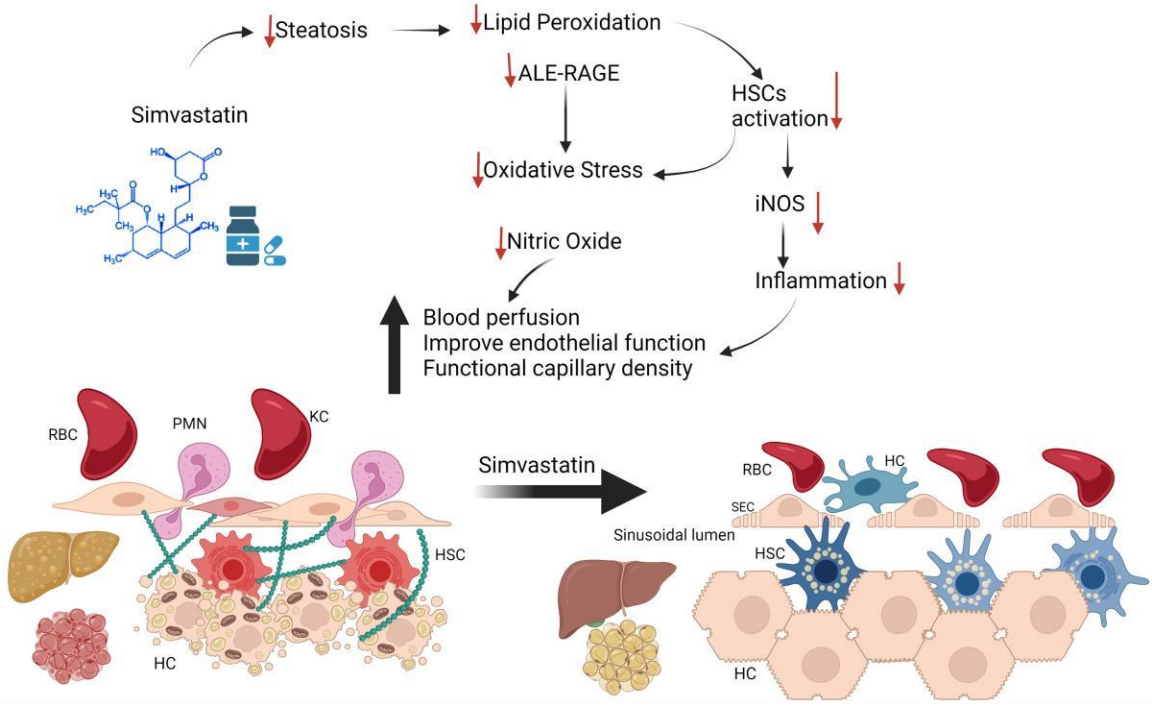

Fig. 2.5 The impact of oral treatment of simvastatin on the development of NAFLD (This image was created by Biorender.com).

Adverse Effects of Statin

The most severe adverse effect of statin therapy is myotoxicity. Its various forms include myopathy, myalgia, myositis and rhabdomyolysis (Joy and Hegele, 2009). However, due to fear that statins will cause harm in patients with high transaminases activity in CVD risk patients was very limited (Athyros *et al*., 2017 and Targher *et al*., 2010). Statins can cause elevations in liver biochemistries and there is a concern that patients with underlying liver disease may beat increased risk for hepatotoxicity (Gazzerro *et al*., 2012). It has been also suggested that long term statin treatment may worsen hepatic histology in patients with NAFLD (Rzouq *et al*., 2010).

Effect of Statins on body weight and glucose

Pereira *et al*. (2022) noted that the high-fat–high-carbohydrate diet fed mice gained more weight than the high-fat–high-carbohydrate diet + simvastatin fed mice also there were significant changes in glucose levels in high-fat–high-carbohydrate diet fed mice when compared to the high-fat–high-carbohydrate diet + simvastatin fed mice and control group. Souza *et al*. (2020) study showed that when the animals were fed with high-fat diet and treated with simvastatin showed decrease in the body mass and relative weights of liver when compared with normolipidic diet fed mice. Li *et al*. (2020) observed decrease in the body weight of simvastatin + HFD fed mice when compared with High-fat diet-fed mice.

Effect of Statins on sero-biochemistry

Panajatovic *et al*. (2021) investigated that simvastatin (SIM) effect on lipid metabolism in mice caused an decrease in plasma cholesterol and triglyceride values, slight increase in HDL cholesterol. In contrast, simvastatin effect on lipid metabolism in mice caused a significant increase in plasma cholesterol, HDL cholesterol and serum triglycerides values. Souza *et al*. (2020) study showed that when the animals were fed with high-fat diet and treated with simvastatin showed decrease in the values of total cholesterol and triglycerides and increase in the values of HDL cholesterol than mice fed with the normolipidic diet. Pereira *et al*. (2022) noted that high-fat–high-carbohydrate fed mice showed significantly increased serum cholesterol and triglycerides, as well as ALT and AST enzyme activity and a reduction in HDL cholesterol, compared to the high-fat–high-carbohydrate +SIM mice and control group mice. Li *et al*. (2020) observed HFD feeding significantly increased the serum lipid levels of TC, TG, and the liver injury biomarkers, such as ALT, AST and decreased HDL levels, whereas the Simvastatin treatment reversed nearly all the above parameters at both examined time-points. Rodrigues *et al*. (2019) and Wang *et al*. (2013) stated noticed that the serum levels of

total cholesterol, triglyceride and AST, ALT activity were significantly increased with the consumption of high-fat diet but the serum levels of total cholesterol, triglyceride and AST, ALT activity were all declined in rats of simvastatin treatment group.

Effect of statins on Antioxidants

Rodrigues *et al*. (2019) noticed that NASH+SIM showed a significant decrease in TBARS value as compared to NASH+ Vehicle. SOD activity was increased

in the treated (NASH+Simvastatin) group compared to that in the NASH+ Vehicle group and the GPx activity increased in the treated (NASH +Simvastatin) group as compared to that in the NASH+Vechicle group. Pereira *et al*. (2022) examined the hepatic and adipose tissue oxidative stress *via* TBARS revealed that lipid peroxidation in high-fat–high-carbohydrate -fed mice was significantly increased compared to that in the control or the high-fat–high-carbohydrate +SIM group. The activity of the antioxidant catalase (CAT) was significantly decreased, while that of superoxide dismutase (SOD) was increased in the liver of high-fat–high-carbohydrate -fed mice, compared to that of the control or

HFHC-treated group. Furthermore, increase in hepatic nitric oxide bioavailability and iNOS protein expression were observed in mice fed with high-fat–high-carbohydrate diet compared to those fed with normal chow or high-fat–high-carbohydrate +SIM mice.

Effect of Statins on Cytokines

Wang *et al*. (2013) noticed that with the progress of NASH, hepatic expression of eNOS both in mRNA and protein levels were gradually decreased, rats treated with simvastatin had higher mRNA expression of eNOS. TGF-β plays a critical role in the initiation, promotion, and progression of HSC activation in rats fed with high-fat diet for 24 weeks, treatment with simvastatin inhibited the activation of TGF-β. Souza *et al*. (2020) study observed an increase in the levels of NF-κB, plasma TNF-*a* in animals fed a high-fat diet, and plasma IL-10 was decreased in animals fed a high-fat diet when compared to the high-fat diet treatment with simvastatin

Effect of Statins on Histopathology

Rodrigues *et al*. (2019) noticed changes in the liver of NASH+Vechile group showing microvesicular steatosis, macrovesicular steatosis, inflammation, and hepatocellular ballooning, Whereas, simvastatin administration markedly attenuated the primary ballooning process in the liver. Pereira *et al*. (2022) noted histologically, steatosis, hepatocyte ballooning, and fibrosis in the livers of high-fat–high-carbohydrate -fed mice, while no inflammatory cell infiltration was observed in the high-fat–high-carbohydrate +SIM mice and control group mice. Wang *et al*. (2013) histopathological observation revealed that steatosis appeared at the end of 8^{th} week, steatohepatitis, hepatocellular ballooning degeneration, inflammatory cells infiltration, spotty focal necrosis, moderate or severe hepatocellular steatosis were established at the end of 16^{th} week and at 24^{th} week all model rats exhibited sinusoidal fibrosis with the consumption of high-fat diet. These conditions were improved in rats of simvastatin treatment group.

LACTOFERRIN

Lactoferrin (LF), an element of the transferrin family, is an iron-binding glycoprotein present in exocrine secretion and serum. LF is produced by the glandular cells, and is present in the saliva, milk, tears, and mucous secretions. It is also found in neutrophils that are released in to the blood and the inflamed infected tissues (Farid *et al*., 2021). Lactoferrin plays a major role in immune responses and defends against various infections. It has antimicrobial activities against bacterial and parasitic infections. It also has an anticancer activity (Farnaud and Evans, 2003 and Actor *et al*., 2009) and anti oxidative property (Kanyshkova, 2001, Legrand, 2005). These activities rely not only on

its capacity to bind iron, but also to interact with molecular and cellular components of both the host and pathogens (Latorre *et al.*, 2010).

Structure of Lactoferrin

Lactoferrin was first discovered and isolated from bovine milk and is a member of the transferrin family (60% amino acid sequence has identity with serum transferrin) LF is a single polypeptide chain glycoprotein with a molecular weight of around 75-80 kDa (Baker *et al.*, 2000).

The amino acid sequence of bovine Lactoferrin (BLF) shows that it consists of a single polypeptide chain of 689 residues (Mead and Tweedie, 1990 and Pierce *et al.*, 1991). Superti (2020) reported that LF as the other transferrins, has a molecular weight of about 80 kDa and its structure includes two lobes, each capable of reversibly chelating two Fe3+ ions per molecule. The polypeptide chain of LF is folded into two symmetrical lobes i.e. N-lobe and C-lobe. Each lobe consists of two domain referred as N1 and N2 in one domain and C1 and C2 are in another domain. Each lobe binds with one metal ion iron (Baker and Baker, 2005). Fleming and Bacon (2005) reported that usually only about 15 percent of LF is saturated with iron, indicating that the two lobes are not entirely occupied by iron. BLF possessing twice the serum transferrin's affinity for iron is also able to act on systemic iron homeostasis by modulating the synthesis of the two key proteins hepcidin and ferroportin through the down-regulation of IL-6.

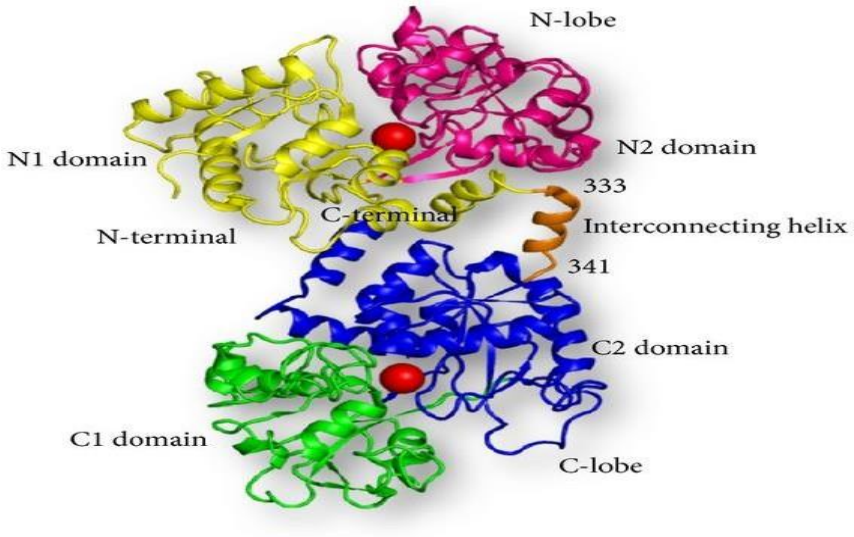

Fig: 2.6 Structure of bovine lactoferrin molecule (PDB code: 1BLF)

The N1 and N2 domains are colored in yellow and pink, respectively, while the C1 and C2 domains are coloured in green and blue, respectively. The interconnecting helix between the lobes is colored in orange. The two iron atoms are shown as red spheres. (Haridas *et al*., 1994, Moore *et al*., 1997 and Sharma *et al*., 2013). The two domains enclose a deep cleft which contains the iron binding site. The N and C-lobes are linked by a three-turn connecting helix, residues 334 to 344, just as in human lactoferrin. This appears to be one of the distinguishing features between lactoferrins and transferrins; the latter contain several prolines in their inter lobe connecting peptides and the conformations are non-helical (Bailey *et al.,* 1988 and Kurokawa *et al.,* 1995).

Lactoferrin in milk is in dissolved state, in aqueous form, it is stabilized by hydrogen bonding, hydrophobic and hydrophilic interactions, disulphide bonds and ligand bindings. These structural stabilizing forces could be affected by pH and heat treatment of

milk. The pH affects the salt bridges and hydrogen bonding whereas heat treatment affects the kinetic energy of hydrogen bonds, nonpolar hydrophobic and intermolecular disulphide interactions (Brisson *et al.,* 2007). Baker and Baker (2004) and Rastogi *et al.* (2016) reported that the LF started losing iron at pH 5.0-6.5 and around 90 percent of its iron was released at pH 2.0. The loss of iron was found to depend on alteration in tertiary structure of LF.

FUNCTION

Lactoferrin (LF) is an 80-kDa glycoprotein present in milk and in other body barrier fluids of vertebrates (Chaneton *et al.,* 2013). Lactoferrin presents antibacterial activity that depends on its iron-chelating activity and on the direct interaction of its cationic amino-terminal region with the bacterial surface (Jenssen and Hancock, 2009). In the bovine mammary gland, LF plays a role in the defense mechanism against bacterial infection, particularly during the dry period, when its concentration increases by 50 fold.

Piccinini *et al*., (2007) and Hiss *et al*., (2009) have reported that the LF concentration is also high during the early days of lactation, which is a finding that has been related to a possible function of LF in preventing the establishment of infection postpartum and in protecting the newborn calf from infections. Cooper *et al.* (2014) reported that LF, a component of the immune system, has antibacterial, anti-inflammatory and immunomodulatory properties. A study by Latorre *et al.* (2012) demonstrated that LF can bind and sequester Lipopolysaccharide, thus preventing activation of the pro-inflammatory pathway, sepsis, and tissue damage. Cooper *et al.* (2014) showed that pigs that were fed with LF resulted in significantly decreased intestinal inflammation. Kuhara

et al. (2014) reported that oral ingestion of LF by mice with hepatitis showed the enhanced expression of IL-11 in the small intestine. Li *et al*. (2012) demonstrated that intraperitoneal injection of LF in mice with LPS-induced acute lung injury had a protective effect.

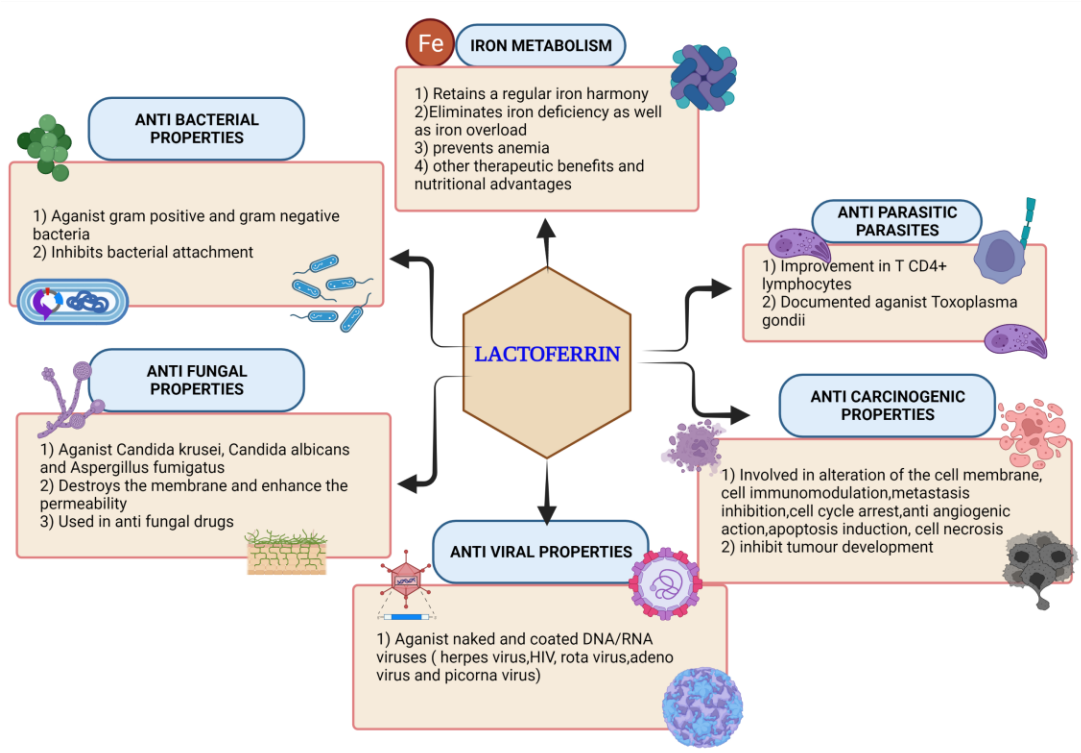

Fig. 2.7 Pharmacological potentials of Lactoferrin against pathological conditions (This image was created by Biorender.com).

Effect of Lactoferrin on Body Weight

Shi *et al*. (2012) reported that when compared to casein, lactoferrin supplementation decreased body weight during energy restriction and suppressed weight regain during the *ad libitum* phase in mice. McManus *et al*. (2015) noticed that there was a significantly decreased body weight in the HFD + BLF group when compared to the

LFD group and HFD group. Min *et al*. (2018) observed at the end of the experiment, treatment with Metformin, lactoferrin, and the combination of the two significantly decreased body weight compared with the HFD group. Aoyama *et al*. (2022) found that there was no significant difference in body weights between Dimethylnitrosamine (DMN) induced control and lactoferrin-treated groups. Ling *et al*. (2019) noticed that after 12 weeks of high-fat/cholesterol feeding, no significant difference was observed in body weight. Sun *et al*. (2016) indicated that receiving bovine LF reduced body weight gain. Xiong *et al*. (2018) noticed that lactoferrin administration has significantly lowered the body weight gain and relative weights of liver in the LF + HFD group than that in the HFD group. Tung *et al*. (2014) noticed that the body weight of the DMN, treated group significantly decreased when compared with the normal control group, and the body weight of the high-dose lactoferrin treated group increased when compared with the DMN treated group.

Effect of Lactoferrin on Glucose

McManus *et al*. (2015) reported that significant increase in plasma glucose level in the HFD group, and there was a decrease in plasma glucose level in the HFD + Lactoferrin treated group. Min *et al*. (2018) noticed that HFD significantly increased fasting blood glucose levels and treatments with metformin, lactoferrin and combination decreased fasting blood glucose levels. Aoyama *et al*. (2022) stated that the level of glucose was significantly higher in the lactoferrin group than that of the control group. Ling *et al*. (2019) noticed that there is significant decrease in blood glucose in high lactoferrin group than in HFD with cholesterol group. Sun *et al*. (2016) indicated that the BLF intervention significantly decreased the blood glucose level when compared to mice

in HFD group. Xiong *et al*. (2018) reported that the lactoferrin supplementation significantly reduced the serum concentration of glucose when compared with the HFD group.

Effect of Lactoferrin on Sero-Biochemistry

Min *et al*. (2018) reported in the HFD group, the serum levels of TG, TC, and LDL significantly decreased in the treatment groups, and the serum HDL level significantly increased in the Metformin and Metformin + LF groups. Aoyama *et al*. (2022) on biochemical analysis of serum indicated that the activity of AST, Cholesterol, and LDL values in the LF treated groups were significantly lower than that in the control group and there was no dose-dependent change in serum ALT, total protein and HDL values. Asghar *et al*, (2019) reported that there was a decrease in serum AST, ALT and bilirubin values of lactoferrin group when compared with control+ CCl_4. Ling *et al*. (2019) compared that with the HFD with cholesterol group, serum cholesterol levels in the medium lactoferrin and high lactoferrin groups significantly decreased where as lactoferrin supplementation did not change the serum levels of triglycerides and HDL. Sun *et al*. (2016) noticed that BLF consumption significantly reduced the concentration of serum total cholesterol and triglycerides. Xiong *et al*. (2018) reported lactoferrin administration induced significant decreases in the serum cholesterol when compared with the HFD group. Chen *et al*. (2016) suggested that both serum AST and ALT activity were significantly lower in mice that had received either a low or a high LF diet than in those mice that received a control or high-cholesterol diet.

Effect of Lactoferrin on Antioxidants

Farid *et al.* (2021) revealed that a significant reduction in the activity of SOD and GPx, and a non significant increase in the level of MDA was observed in the CCl_4 group when compared with the control group. The LF group showed a significant increase in the level of SOD, and a non-significant difference in the level of GPx and MDA when compared to the control group. Chen *et al*. (2016) noticed that GSH activity in liver sample that were fed on either a low or a high LF diet were significantly higher than in those mice that received a control or high-cholesterol diet. Malondialdehyde (MDA) levels were significantly lower in mice that had received either a low or a high LF diet than in those mice that received a control or high-cholesterol diet.

Effect of Lactoferrin on Cytokines

Aoyama *et al*. (2022) the inflammatory cytokines, IL-6, TNF-*a*, NF-*κ*B, IL-18, and IL-1 β, were significantly down-regulated by LF compared with the control, The mRNA expression of TGF- β as fibrosis-related cytokines were significantly down-regulated by LF. Farid *et al*. (2021) reported a significant decrease of IL-10 value in the CCl_4, LF-protected, and LF-treated groups in comparison with the control group. Sun *et al*. (2016) observed that the BLF supplementation significantly decreased the expression of proinflammatory cytokine like TNF-*a*, when compared with HFD fed group. Xiong *et al*. (2018) concluded that there is no significant difference between the HFD group and LF + HFD group in the NF-*κ*B, IL-6 and TNF-α mRNA levels in the liver. Chen *et al*. (2016) observed a significant reduction in expressions of TGF- β were detected in livers of mice that had received a LF diet, rather than in those mice that had received a control diet or high-cholesterol diet and significantly lower expressions of TNF- α and IL-1 β

were also detected in livers of mice that had received a LF diet, but not in those mice that had received a control diet or high cholesterol diet.

Effect of Lactoferrin on Histopathology

Shi *et al*. (2012) observed in obese mice fed *ad libitum* with FFD, the liver histology showed variable degrees of fat accumulation ranging from a focal midzonal and perivenular vesicle clusters to diffuse macrovesicular fatty changes, but fibrosis was absent, the livers of the LF supplementation group showed less steatosis and improved tissue morphology. Min *et al*. (2018) observed that HFD showed enlarged adipocyte size which was reduced by the metformin + LF group. Aoyama *et al*. (2022) in H&E staining observed the livers of HFD and DMN treatment groups showed diffuse deposits of fat droplets with hepatocellular ballooning and neutrophil infiltration in the lobule, whereas lactoferrin treatment significantly reduced fat deposition, lobular inflammation, and ballooning injury of hepatocytes in a dose-dependent manner. Farid *et al*. (2021) noticed that the CCl_4 treated group showed a diffused ballooning degeneration of the hepatocytes of the parenchyma and it was associated with congestion in the portal vein. However, in the LF-protected group, there was no histological alteration. Ling *et al*. (2019) observed in the H&E stained sections that the liver cell cords in the high fat diet with cholesterol group were disordered with fatty degeneration and inflammatory cell infiltration, the sections stained by Oil Red O revealed that liver lipid droplets were decreased by lactoferrin treatment. H&E staining demonstrated that HFD feeding induced the fat balloon-like structure and inflammatory cell infiltration, while BLF intervention suppressed these abnormalities reported by Sun *et al*. (2016). Tung *et al*. (2014) demonstrated that DMN induced liver injury, causes fatty degeneration and necrosis in

the central vein where as the levels of fatty degeneration and necrosis was significantly reduced with lactoferrin treatment.

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CHAPTER III

MATERIALS AND METHODS

The experimental study was conducted in healthy adult male mice of 6-7 weeks age and weighing about 28 g on an average. The animals were procured from VYAS Labs, Hyderabad (CPCSEA: 2085/PO/Rc.Bi.Bt/S/19/CPCSEA). The mice strains used for the study was C57BL/6 a classic murine model for experimental NAFLD and were reared under uniform environmental conditions with a temperature range of 22±2ºC for about 6 weeks with an automatically controlled photoperiod (12 hr light –12hr dark cycle). Mice were provided with standard diet *ad libitum* along with free access to clean drinking water throughout the experiment period. Prior to the beginning of trial, the animals were acclimatized for a period of 15 days.

The protocols adopted in this experimental study were approved by the Institutional Animal Ethics Committee (IAEC), College of Veterinary Science, Hyderabad (IAEC, Approval No. CPCSEA 1/24/C.V.Sc, Hyd, IAEC.MICE/ dated 12.06.2021) and is in accordance with the guidelines for the Care and Use of Laboratory Animals published by the US National Institute of Health.

MATERIALS

Drugs

Name	Source
Carbon Tetra Chloride	Sigma Aldrich, USA
Bovine Lactoferrin	Bioven Ingredients, India
Simvastatin (Simvotin^{TM}, 10 mg)	Sun pharmaceutical Ind Ltd, India

Chemicals and Kits

All chemicals used in biochemical analysis were of analytical grade.

1. All the chemicals (for preparation of reagents and buffers) were procured from Sigma-Aldrich, USA, Qualigens Pvt. Ltd. Mumbai, Himedia Pvt. Ltd., Mumbai and SRL Pvt. Ltd., Mumbai.
2. Kits for total cholesterol, triglycerides, HDL and glucose were procured from ERBA diagnostics Ltd, Surat, India.
3. Kits for Aspartate transaminase (AST), alanine transaminase (ALT), total protein, total bilirubin and GGT were procured from ERBA diagnostics Ltd, Surat, India.
4. The enzyme linked immunosorbent assay (ELISA) kits for IL-10, TNF-α, NF-κB and TGF-β were procured from Krishgen Biosystems, Mumbai, India.

Feed

a) Standard pellet feed for mice was procured from M/s. VRK Nutritional solutions, Hyderabad.

b) High Fat Diet in the form of brown coloured pellets produced from starch, casien, skim milk powder, lard, minerals and vitamins was procured from M/s. VRK Nutritional solutions, Hyderabad.

Composition of High Fat Diet (HFD):

Moisture	2.15%
Protein	18.05%
Fat	40.20%
Fiber	2.65%

Calcium	1.20%
Phosphorus	0.65%
Total Ash	4.00%
Aflatoxin	Not present
Salmonella	Not present
E.coli	Not present

Instruments

a. Tissue homogenizer with speed regulators – Remi motors Ltd.

b. Ultrasonic cleaner – Citizon- India

c. Ultrasonic homogenizer box and Sound proof box – Athena, Mumbai, India

d. Dry bath SLM-DB-120 – Precision Sciences, Bangalore, India

e. Micropipettes of 0.5-2.5 µl, 2-20 µl, 100 µl, 200 µl, 1000 µl – Eppendorf

f. Multichannel pipette

g. Microplate Reader- i Mark™ - Bio-rad

h. Digital pH meter – 335 – Thoshniwal

i. Digital electronic balance– Shimadzu, Japan

j. Digital electronic balance– Aczel, India

k. Magnetic stirrer – Remi eletronics Ltd, Mumbai, India

l. Gel rocker

m. Vortex mixer – Lead instruments

n. Laboratory freezing centrifuge R8C- Remi eletronics Ltd, Mumbai, India

o. Ultracentrifuge – Remi eletronics Ltd, Mumbai, India

p. Deep freezer (-20^{0}C) - Blue star

q. Ultra – low deep freezer (-80^{0}C) – Thermofischer scientific, USA

r. Spinwin MC - Tarsons

s. U.V. Microscope-Olympus AX 70, Japan

Stains:

Stains and chemicals for the histopathological study of liver tissue were obtained from M/s. Qualigens Pvt. Ltd., Mumbai.

METHODS

Animals

A total of 44 male C57BL/6 mice of 6-7 weeks age were procured and acclimatized for 15 days before beginning the study. The mice were randomly divided into 6 groups consisting of 6 mice in group 1 and 3 where as group 2, 4, 5 and 6 had 8 animals each. For the purpose of statistical analysis (n=6) has been taken. The animals were kept in polypropylene cages and maintained with 12 hr dark/light cycle under hygienic conditions having ambient temperature (22–24^{0}C) at college animal house in Department of Veterinary Pharmacology and Toxicology. Control group animals were fed with commercial standard pellet feed and the model group animals were fed with High Fat Diet and water *ad libitum* throughout the experiment. Millipore (reverse osmosis) water was employed for oral gavage. All the groups were maintained as per the treatment schedule for 6 weeks.

Experimental Design

Group	Treatments	Number of animals
1	Standard diet	06
2	HFD + CCl_4 @ 0.5mg/Kg b.wt in olive oil i.p twice in a week	08
3	BLF @300mg/Kg b.wt p.o	06
4	HFD +CCl_4 @ 0.5mg/Kg b.wt in olive oil i.p twice in a week + BLF @ 300mg/Kg b.wt p.o)	08
5	HFD + CCl_4 @ 0.5mg/Kg b.wt in olive oil i.p twice in a week + BLF @ 100mg/Kg b.wt p.o)	08
6	HFD + CCl_4 @ 0.5mg/Kg b.wt in olive oil i.p twice in a week +Standard drug (Simvastatin @ 10mg/Kg b.wt p.o)	08

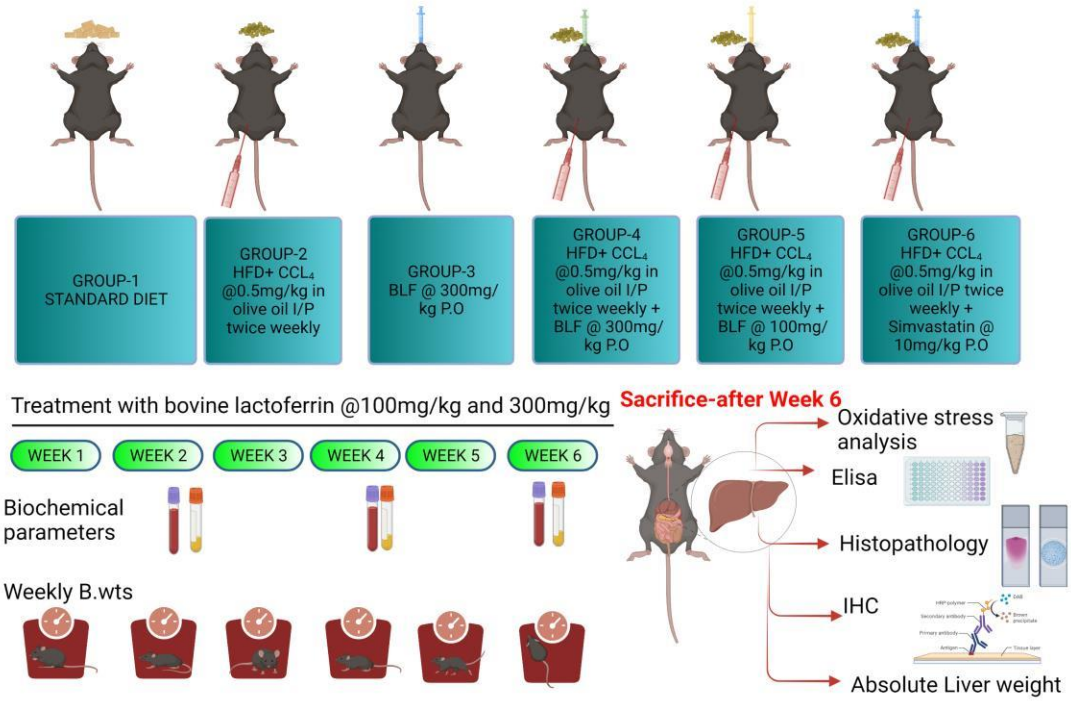

Fig. 3.1 Experimental design and intervention of Bovine lactoferrin in CCl_4 & HFD-induced NAFLD (This figure was created with Biorender.com.)

Body Weights: Individual body weights of all the mice were recorded on 0^{th}, 1^{st}, 2^{nd}, 3^{rd}, 4^{th}, 5^{th} and 6^{th} week of experiment.

Liver Weights: Absolute liver weights were recorded on sacrifice of the animals.

Blood Collection

Blood collection was carried out on 2^{nd}, 4^{th} and 6^{th} wk. Feed was withdrawn 12 hr before the blood collection and blood was collected through retro-orbital plexus. Sera samples were separated from the blood for the estimation of Glucose, lipid profile. The serum samples were analyzed for the activity of TC, TG, HDL, Glucose, AST, ALT, TP, TBR and GGT.

After 6^{th} week of blood collection, mice were euthanized by carbon dioxide exposure by using CO_2 chamber and liver tissue were collected and weighed. Liver tissue was homoginized and supernatant was stored at -80^0C for further estimation of pro-inflammatory and Anti-inflammatory cytokines like IL-10, TNF-α, NF- κB and TGF-β as well as oxidative stress parameters like TBARS, GSH, Nitric oxide estimation, and SOD activity were analysed in liver tissue. Furthermore, liver tissue was collected in 10 percent neutral buffered formalin (NBF) for the histological examination to draw possible conclusions.

Preparation of Tissue Homogenates

Animals were euthanized using CO_2 chamber after 6^{th} week, liver tissues were removed immediately and made into four parts for analysis of various parameters like oxidative stress, ELISA, immunohistochemistry and histopathology. The tissue samples used for oxidative stress were homogenized in a solution of ice-cold PBS buffer (pH 7.4)

and tissue samples for ELISA were homogenized in a Tris-Tritone buffer (pH 7.4) at 4^0C.

Biochemical Profile

Serum was separated from the blood and used for biochemical analysis at the end for the following parameters by using commercially available diagnostic kits:

1. Total cholesterol
2. Triglyceride
3. High density lipoprotein (HDL) cholesterol
4. Glucose
5. Aspartate transaminase (AST)
6. Alanine transaminase (ALT)
7. Total Protein (TP)
8. Total Bilirubin (TBR)
9. *Gamma*-glutamyl transferase (GGT)

Organ Antioxidant Profile (Liver)

Estimation of Protein (Lowry *et al*., 1951)

Principle**:**

Proteins react with copper at an alkaline pH to form copper-protein complex. This complex, when treated with phosphomolybdic-phosphotungstic reagent (Folin-ciocalteu), forms blue color which is measured at 660nm.

Procedure:

100µL of homogenate was made up to 1.0 mL with distilled water. To this, 5mL of freshly prepared alkaline copper sulphate solution (a mixture of 50mL of 2% sodium carbonate in 0.1N sodium hydroxide and 1.0mL of copper sulphate in 1% potassium

sodium tartarate) was added and kept for 10 min at room temperature. 0.5mL of Folin-ciocalteu reagent was added and allowed to stand at dark for 30 min. The resultant blue color was read at 660nm. Bovine serum albumin (BSA) was used as standard.

Thiobarbituric Acid Reacting Substances (TBARS): (Balasubramanian *et al*., 1988).

Principle:

Malondialdehyde (MDA), formed from the breakdown of polyunsaturated fatty acids, serves as a convenient index for determining the extent of peroxidation reaction. Malondialdehyde has been identified as the product of lipid peroxidation that reacts with thiobarbituric acid to give a red colour which absorbs maximum light at 535 nm.

Reagents:

- 10% Trichloroacetic acid
  - Trichloroacetic acid : 10 gm
  - Distilled water : 100 mL
- 0.67% Thiobarbituric acid
  - Thiobarbituric acid : 0.67gm
  - Distilled water : 100 mL
- 0.2 M TrisHCl buffer pH 7.2 (Homogenizing buffer)

A. Tris (hydroxyl) aminomethane - 1.2114 gm in 50 mL distilled water

B. Concentrated HCl - 0.858 mL in 50 mL distilled water

To 50 mL of solution A, 44.2 mL of solution B was added and diluted to a total

of 200 mL with distilled water.

Procedure

1. 1gm of liver tissue sample along with 10 mL of 0.2 M Tris HCl buffer (pH 7.2) was taken in a tissue homogenizer to get a 10% homogenate.
2. 500µL of supernatant from the homogenate, 1mL of 10% trichloroacetic acid and 1mL of 0.67% thiobarbituric acid were taken in a tightly stoppered tube.
3. The tube was heated to boiling temperature for 45 min.
4. After cooling the tube, the contents were centrifuged.
5. The supernatant was read at 532 nm against blank.
6. The concentration of test samples was obtained using molar extinction coefficient of MDA.
7. From the tissue homogenate, total protein content was estimated by Lowry method (Lowry *et al*., 1951).

Units of activity:

Units of activity = n moles of MDA / mg of protein

Calculation:

$$\text{n moles of MDA / mg of protein} = \frac{2 \times \text{volume of sample taken} \times \text{absorbance of sample}}{1.56 \times 10^5 \times \text{mg of protein}} \times 10^9$$

Reduced Glutathione (GSH) (Moron *et al.*, 1979)

Principle

The method is based on reaction of reduced glutathione (GSH) with 5-5' dithiobis-2-nitrobenzoic acid (DTNB) which gives a compound that absorbs light at 412 nm.

Reagents:

- 25% Trichloroacetic acid (TCA)
  - Trichloroacetic acid : 25 gm
  - Distilled water : 100 mL
- 0.6 mM DTNB (5- 5' dithiobis-2-nitrobenzoic acid) in 0.2 M Sodium phosphate (pH 8.0)
  - Sodium phosphate : 2.839 gm
  - Distilled water : 100 mL

  Adjusted to desired pH and then added
  - DTNB : 23.7 mg
- 0.2 M Phosphate buffer (pH 8.0)
- Solution A: 0.2 M KH_2PO_4: 1.36 gm KH_2PO_4 in 50 mL distilled water
- Solution B: 0.2 M NaOH: 0.4 gm NaOH in 50 mL distilled water

Solution A was taken into a beaker and titrated with solution B till desired pH was obtained and made the volume up to 100 mL with distilled water and stored at 4^0C till usage.

Procedure:

To 400 µL of liver tissue homogenate, 100 µL of 25% trichloroacetic acid was added and centrifuged. Supernatant was collected and used as sample. To 2.0 mL of 0.6 mM DTNB in 0.2 M sodium phosphate (pH 8), 0.1mL of sample and 0.9 mL of 0.2 M phosphate buffer was added and the absorbance was read at 412 nm against a reagent

blank. The standards (0.05-5 mg/mL) were also treated in the same way.

Units of activity:

The values were expressed as n moles of GSH / mg of protein

Estimation of Catalase activity (Asru, 1972)Principle:

Catalase containing sample is allowed to split H_2O_2 followed by addition of dichromate / acetic acid mixture to stop the reaction. Dichromate in acetic acid is reduced first to unstable blue colored perchromic acid and finally to stable green coloured chromic acetate in the presence of H_2O_2, which is measured colorimetrically at 570 nm. Thus measured chromic acetate gives the amount of freely available H_2O_2.

Procedure:

To assay mixture containing 0.4 mL of 0.2 M H_2O_2 and 0.5 mL of 0.01 M phosphate buffer (pH 7), 0.1 mL of liver tissue homogenate was added and mixed well. To this, 2 mL of dichromate acetic acid solution was added exactly after 60 sec. and kept in boiling water bath for 10 min. The absorbance of green coloured chromic acetate formed was measured at 570 nm against reagent blank containing 0.4 mL of 0.2 M H_2O_2 and 0.5 mL of 0.01 M phosphate buffer (pH 7.0).

Units of activity:

The enzyme activity was expressed as µg of H_2O_2 decomposed/min /mg of protein.

Super Oxide Dismutase (SOD) (Madesh and Balasubramanian, 1998)

Principle:

This reaction involves generation of superoxide by pyrogallol autooxidation and the inhibition of superoxide dependent reduction of the tetrazolium dye MTT [3- (4-5 dimethyl thiazol 2-xl) 2, 5-diphenyl tetrazolium bromide] to its formazan, read at 570 nm. The reaction is terminated by the addition of dimethyl sulfoxide (DMSO), which helps to solubilize the formazan formed. The colour evolved is stable for hours and is expressed as SOD units (one unit of SOD is the amount in mg of protein required to inhibit the MTT reduction by 50 %).

Reagents:

- Pyrogallol (100 µM): 6.3 mg of pyrogallol was dissolved in 5 mL of distilled water. One ml from this solution was diluted to 100 mL with distilled water.
- MTT (1.25 mM): 2.58 mg of MTT was dissolved in 5 mL of distilled water.
- Phosphate buffer saline (PBS): PBS was prepared by dissolving NaCl (8 gm), KCl (0.2 gm), KH_2PO_4 (0.2 gm) and Na_2PO_4 (0.94 gm) in 800 mL of distilled water. The pH was adjusted to 7.4 and the volume was made up to 1 liter with distilled water.

Procedure:

The reagents were added separately to the sample, control and the blank tubes as shown below. The absorbance was read at 570 nm against distilled water (blank)

Reagents	Sample	Control	Blank (Duplicate)
PBS	0.65 mL	0.65 mL	0.65 mL
MTT	30 µL	30 µL	30 µL
Homogenate	10 µL	-	-
Pyrogallol	75 µL	75 µL	75 µL

The sample, control and blank tubes were incubated for 5 min at room temperature

DMSO	0.75 mL	0.75 mL	0.75 mL
Homogenate	-	10µL	-

Total protein content of tissue homogenate was estimated by Lowry method (Lowry *et al*., 1951).

Calculation:

Superoxide dismutase was expressed as SOD units/ mg of protein

$$Y\ \% = \frac{\text{Absorbance of sample}}{\text{Absorbance of standard}} \times 100$$

$$\text{SOD (Units/mg of protein)} = \frac{\text{mg of protein in 0.01 mL of tissue homogenate}}{Y\%} \times 50 \times 100$$

Estimation of Nitric Oxide Levels (Miranda *et al.,* 2001)

Principle

Under aerobic conditions, nitric oxide reacts with oxygen to produce stable products (nitrate and nitrite). Scavengers of nitric oxide compete with oxygen leading to production of nitrite ions, which can be quantified using Griess reagent.

Reagents

a) Vanadium chloride (8 mg/mL of 1M HCl)

b) Griess reagent: 0.1% W/V of NEDD (N-(1-naphthyl) ethylenediamine dihydrochloride) and 2% sulfanilamide in 5% HCl.

Procedure

1. 100 µL of liver tissue homogenate was added in 96 well plate.
2. 100 µL of vanadium chloride was added.
3. 100 µL of Griess reagent was added immediately and incubated at 370 C for 20-30 min and the absorbance was recorded at 540 nm.
4. A standard curve was performed using 0.5-25 µM sodium nitrite for calibration.

Units of activity

The values obtained were expressed as µM of nitrate per mg of tissue.

Cytokine Profile

Tumour Necrosis Factor – *Alpha* (TNF-α): The assay employs a quantitative sandwich enzyme immunoassay technique to measure TNF-α. The ELISA kit was procured from Krishgen Biosystems, Mumbai, India.

Interleukin-10 (IL-10): The assay employs a quantitative sandwich enzyme immunoassay technique to measure IL-10. ELISA kit was procured from Krishgen Biosystems, Mumbai, India.

Nuclear Factor - *Kappa* B (NF-κB): The assay employs a quantitative sandwich enzyme immunoassay technique to measure NF-κB. The ELISA kit was procured from Krishgen Biosystems, Mumbai, India.

Transforming Growth Factor (TGF-β): The assay employs a quantitative sandwich enzyme immunoassay technique to measure TGF-β. ELISA kit was procured from Krishgen Biosystems, Mumbai, India.

3.3. HISTOPATHOLOGICAL STUDIES

Tissues pieces of liver were collected from the mice that were sacrificed at the end of the study and fixed in 10% neutral buffered formalin (NBF). The fixed tissues were processed, sectioned (5μm) and stained with routine haematoxylin and eosin (H & E), Masson's trichome stain (MTS) and Oil red O stain for histopathological examination as per the standard procedure. (Luna, 1968).

IMMUNOHISTOCHEMICAL ANALYSIS OF BCL-2 PROTEIN IN THE LIVER

Immunohistochemistry was performed on 5 μm thick sections after de-paraffinisation at 60^0C for 20min (Kumar *et al*., 2014).

i. Immersed in xylene for 5 min (twice).
ii. Hydration step was done in alcohol of different grades - 100%, 90%, 80% and 70% in descending order for 3 min in each.
iii. Slide was rinsed with 1x TBS.
iv. Specimen was covered with 20 μg/mL proteinase - K and incubated at RT for 20 min at 37^0C.
v. Slide was rinsed with 1x TBS.
vi. Slides were mounted with 3% H_2O_2 by using cover slip for 10-15 min.
vii. Washed with 1x TBS for 5 min.
viii. Slides were blocked with 3% bovine serum albumin (BSA).
ix. Slides were Incubated with primary antibody (BCL-2 in 1:200 dilution) overnight and covered with paraffin tape and kept at 4^0C.
x. Slides were washed with TBS-T for 10 min for 3 times.
xi. Slides were mounted with poly excel horse radish peroxidase (HRP) target binder for 30 min.
xii. Slides were washed with TBS-T twice for 10 min each.
xiii. Slides were mounted with 3, 3-o-diaminobenzidine (DAB) staining for 2-10 min.
xiv. Slides were washed with millipore water for 3 times.
xv. Counter stain haematoxylin was added and incubated for 2 min.

xvi. Dehydration step was done by using ascending order of alcohol percentage for 3min and then once in xylene for 5 min.

xvii. Slides were mounted with dibutylphthalate polystyrene xylene (DPx).

STATISTICAL ANALYSIS

The data were subjected to statistical analysis by applying one way ANOVA using statistical package for social sciences (SPSS) version 25.0. Differences between means were tested using with Duncan's post hoc test and significance level was set at $p < 0.05$.

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CHAPTER IV

RESULTS

An experimental study was conducted on male C57BL/6 mice to evaluate the therapeutic efficacy of BLF in NAFLD induced by CCl_4 and HFD. The results of the study were presented as below:

BODY AND ORGAN WEIGHTS

Average Body Weight

The average body weights (g) of mice at the beginning of the experiment were from 28.45±1.68 to 28.66±4.33 as there was no significant difference observed among all groups. The average body weights (g) of group 2 was significantly ($p<0.05$) higher (ranged from 26.86±9.14 to 36.15±6.14 than of group 1 mice (ranged from 29.03±2.19 to 32.53±2.38) from 1st week to 6th week of the experimental period when compared to treatment groups 4 to 6. There was a significant ($p<0.05$) decrease in average body weights (g) from group 2 to group 6 on 1st & 2nd week of experiment. All the treatment groups showed a significant ($p<0.05$) increase in average body weights (g) from 3rd week of treatment when compared to group 3. The average body weights (g) of group 3(ranged from 28.93±2.08 to 31.51±1.10) showed a significant ($p<0.05$) decrease when compared with group 1(ranged from 29.03±2.19 to 32.53±2.38) throughout the experimental period (Table 4.1; Fig. 4.1).

Average Absolute Liver Weight

The average absolute weight of liver (g) in group 2 (2.12±1.31) was significantly ($p<0.05$) increased in the 6th week, when compared to the group 1& 3 (1.41±0.41 & 1.33±0.36), while treatment groups 4, 5 (1.47±0.51 & 1.56±0.93) and 6 (1.54±0.86) showed significant ($p<0.05$) decrease in liver weight in comparison to group 2. The value of group 4 (1.47±0.51) was comparable to group 1 (Table 4.2; Fig. 4.2).

Relative Liver Weight

The relative weight of liver (%) in group 2 (5.86±0.29) was significantly ($p<0.05$) increased in the 6th week, when compared to the group 1& 3 (4.33±0.05 & 4.22±0.08), meanwhile in treatment groups 4, 5 (4.43±0.06 & 4.56±0.04) and 6 (4.51±0.04), there was a significant ($p<0.05$) change in relative liver weight when compared to the group 2. The value of group 4 (4.43±0.06) was comparable to group 1 (Table 4.2; Fig. 4.3).

SERUM BIOCHEMISTRY

Total Cholesterol

On 2nd & 4th week of the study, the concentration of total cholesterol (mg/dL) showed a significant ($p<0.05$) increase in groups 2, 4, 5 and 6 as compared to groups 1 and 3. In the 6th week group 2 (203.66±8.99) showed a significant ($p<0.05$) increase when compared to groups 4,5 and 6, whereas in the 6th week the treatment groups 4 and 5 (116.08±2.78 and 123.03±2.89, respectively) showed a significant ($p<0.05$) increase in total cholesterol concentration when compared to group 6 (98.31±3.06). The total cholesterol concentration of group 3 (from 89.03±2.61 to 86.78±3.50) showed a significant ($p<0.05$) decrease when compared with group 1 (from 92.90±1.26 to 94.50±1.70) on 4th and 6th week of the experimental period (Table 4.3; Fig. 4.4).

Triglycerides

The concentration of triglycerides (mg/dL) showed a significant ($p<0.05$) increase in groups 2, 4, 5 and 6 as compared to groups 1 and 3. In the 6th week group, 2 (121.95±11.84) showed a significant ($p<0.05$) increase when compared to groups 4, 5 and 6, whereas on 6th week the treatment groups 4 and 5 (71.06±7.84 and 73.91±6.37, respectively) showed a significant ($p<0.05$) increase in triglyceride concentration when compared to group 6 (68.00±6.85). The triglyceride concentration of group 3(from 52.63±6.38 to 49.03±5.99) showed a significant ($p<0.05$) decrease when compared with group 1(from 62.03±3.29 to 66.03±2.37) on 4th and 6th week of the experimental period (Table 4.4; Fig. 4.5).

HDL Cholesterol

The concentration of HDL cholesterol (mg/dL) on 2nd week, showed a significant ($p<0.05$) decrease in groups 2, 4, 5 and 6 (30.76±0.55, 42.21±1.04, 39.15±0.92 and 43.18±0.97, respectively) when compared to groups 1 and 3 (49.36±1.25 and 50.40±1.02, respectively), whereas on 4th week the treatment groups 4, 5 and 6 (48.96±2.05, 46.03±1.24 and 51.11±1.02) showed significant ($p<0.05$) increase when compared with group 2(23.55±0.70). On 6th week, the HDL of group 2 (16.80±0.67) was decreased significantly ($p<0.05$) when compared to all other groups (Table 4.5; Fig. 4.6).

Glucose

The concentration of glucose (mg/dL) of group 1 was significantly ($p<0.05$) lower (ranged from ranged from 241.66± 1.37 to 250.50± 2.47) and that of group 2 was significantly ($p<0.05$) higher (ranged from 287.66±8.81 to 310.33±5.79) throughout the experimental period when compared to treatment groups 4 to 6. All the treatment groups

showed a significant ($p<0.05$) decrease in blood glucose concentration from 2nd week of treatment when compared to group 2. The blood glucose concentration of group 3 (ranged from 239.16± 1.33to 231.83± 2.15) showed a significant ($p<0.05$) decrease when compared with group 1(ranged from 241.66± 1.37 to 250.50± 2.47) throughout the experimental period (Table 4.6; Fig. 4.7).

Alanine Transaminase (ALT)

The levels of serum ALT (IU/L) of group 1 was significantly ($p<0.05$) lower (ranged from 40.68±1.4 to 45.23±1.20) and that of group 2 was significantly ($p<0.05$) higher (ranged 204.16±1.79 to 244.18±1.13) throughout the experimental period when compared to treatment groups 4 to 6. All the treatment groups showed a significant ($p<0.05$) decrease in the levels of serum ALT from 2nd to 6th week of treatment when compared to group 2. The levels of serum ALT of group 3 (ranged from 39.31±1.40 to 34.03±1.46) showed a significant ($p<0.05$) decrease when compared with group 1 (ranged from 40.68±1.4 to 45.23±1.20) throughout the experimental period (Table 4.7; Fig. 4.8).

Aspertate Transaminase (AST)

The levels of serum AST (IU/L) of group 1 was significantly ($p<0.05$) lower (ranged from 121.15±0.47 to 128.11±2.11) and that of group 2 was significantly ($p<0.05$) higher (ranged 213.93±1.21 to 264.93±1.23) throughout the experimental period when compared to treatment groups 4 to 6. All the treatment groups showed a significant ($p<0.05$) decrease in the levels of serum AST from 2nd to 6th week of treatment when compared to group 2. The levels of serum AST of group 3 (ranged from 120.98±1.65 to 117.06±1.48) showed a significant ($p<0.05$) decrease when compared with group 1(ranged from 121.15±0.47 to 128.11±2.11) throughout the experimental period (Table 4.8; Fig. 4.9).

Total Protein Concentration

The Total Protein concentration (g/dL) (Mean±SE) in all the treatment groups 4, 5 and 6 showed a significant ($p<0.05$) increase from 2nd to 6th week (ranged from 6.58±0.02 to 7.40±0.07, 5.56±0.02 to 6.91±0.08 and 6.28±0.03 to 7.02±0.09) when compared to group 2 (ranged from 4.59±0..06 to 4.43±0.05). The total protein concentration (g/dL) of group 3 (ranged from 7.94±0.03 to 8.12±0.09) showed a significant ($p<0.05$) increase when compared with group 1(ranged from 7.57±0.07 to 7.55±0.09) throughout the experimental period (Table 4.9; Fig. 4.10)

Total Bilirubin Concentration

The total bilirubin concentration (mg/dL) (Mean±SE) in all the treatment groups 4,5 and 6 showed a significant ($p<0.05$) decrease from 2nd to 6th week (ranged from 0.19±0.01 to 0.17±0.01, 0.21±0.01 to 0.19±0.01 and 0.21±0.01 to 0.19±0.01) when compared to group 2 (ranged from 0.24±0.01 to 0.38±0.01). The total bilirubin concentration (mg/dL) of group 3 (ranged from 0.11±0.01 to 0.13±0.01) showed a significant ($p<0.05$) decrease when compared with group 1(ranged from 0.13±0.01 to 0.15±0.02) throughout the experimental period (Table 4.10;Fig. 4.11)

***Gamma*-Glutamyl Transferase (GGT)**

The levels of serum *gamma*-glutamyl transferase (GGT) (U/L) (Mean±SE) of all the treatment groups 4,5 and 6 showed a significant ($p<0.05$) decrease from 2nd to 6th week (ranged from 12.62±1.30 to 6.53±0.51, 13.62±0.80 to 8.94±0.43 and 12.66±1.70 to 6.61±0.98) when compared to group 2 (ranged from 16.67±0.8 to 21.80±0.19). The levels of serum

GGT (U/L) of group 3 (ranged from 5.08±1.15 to 5.42±0.30) showed a significant ($p<0.05$) decrease when compared with group 1 (ranged from 5.64±0.12 to 5.92±0.21) throughout the experimental period (Table 4.11;Fig. 4.12).

Antioxidant Profile

Reduced Glutathione (GSH)

The concentration of GSH (nmoles /mg protein) (Mean±SE) was measured in the liver at the end of experiment. The concentration GSH in liver on 6th week revealed a significant reduction in group 2 (14.98±0.44) when compared to all other groups. There was a significant ($p<0.05$) decrease of GSH level in groups 4 (23.15±0.67), 5 (21.24±0.39) and 6 (22.91±0.31) as compared to groups 1 (24.73±1.16) and 3 (24.81±0.54) (Table 4.12; Fig. 4.13).

Catalase (CAT)

The activity of CAT (U/mg protein) (Mean±SE) was measured in the liver at the end of experiment. The activity of CAT in the liver on 6^{th} week revealed a significant ($p<0.05$) reduction in group 2 (43.75±4.10) when compared to all other groups. There was a significant ($p<0.05$) decrease of CAT in group 4 (68.91±3.23), 5(65.78±3.00) and 6 (67.66±3.43) as compared to groups 1 (71.75±4.80) and 3 (72.69±5.92) (Table 4.13; Fig. 4.14).

Super Oxide Dismutase (SOD) Activity

The activity of SOD (U/mg of protein) (Mean±SE) was measured in the liver on 6^{th} week. The activity of SOD in the liver on 6^{th} week revealed a significant

($p<0.05$) reduction in group 2 (3.47±0.11) when compared to all other groups. There was a

significant ($p<0.05$) decrease in the activity of SOD in groups 4 (5.87±0.05), 5 (5.12±0.02) and 6 (5.74±0.05) when compared to groups 1 (6.99±0.29) and 3 (6.26±0.05) (Table 4.13; Fig. 4.15).

Lipid Peroxidation Profile

Thiobarbituric Acid Reacting Substances (TBARS)

The concentration of TBARS (n moles of MDA released/mg protein) (Mean±SE) was measured in the liver at the end of experiment. The concentration of TBARS in liver on 6^{th} week revealed a significant ($p<0.05$) increase in group 2 (3.93±0.04) when compared

to all other groups. There was a significant ($p<0.05$) increase in TBARS in groups 4 (2.48±0.04), 5 (2.95±0.06) and 6 (2.67±0.02) as compared to group 1 (2.19±0.08) and 3 (2.09±0.10) (Table 4.12; Fig. 4.16).

NITRIC OXIDE (NO) CONCENTRATION

The concentration of NO (µM of nitrite/mg tissue) (Mean±SE) in liver revealed a significant ($p < 0.05$) increase in group 2 (8.46±0.72) when compared to the group 1 (2.75±0.13), whereas in treatment group 4, 5 and 6 (3.28±0.21, 4.17±0.16 and 3.18±0.17 respectively) showed a significant ($p < 0.05$) decrease as compared to group 2. The value of group 3 (2.58±0.18) was comparable to group 1 (Table 4.13; Fig. 4.15).

INFLAMMATORY CYTOKINE MARKERS

Interleukin-10 (IL-10)

The IL-10 (pg/mg tissue) in liver homogenate revealed a significant ($p < 0.05$) decrease in group 2 (55.45±0.30) as compared to group 1 (71.61±0.42). The treatment groups 4 (65.54±0.37),5(61.62±0.36) and 6 (64.69±0.32) showed a significant ($p < 0.05$) increase in IL-10 concentration to group 2, but significant ($p< 0.05$) decreased when compared to group 1 (71.61±0.42) and group 3 (69.68±0.33). (Table 4.15; Fig. 4.18).

Tumor Necrosis Factor (TNF-α)

The TNF-α (pg/mg tissue) in liver homogenate revealed a significant ($p < 0.05$) rise in group 2 (52.65±3.45) as compared to group 1 (26.62±2.21). The treatment groups 4 (28.67±2.23),5(36.87±2.05) and 6 (27.73±2.34) showed a significant ($p < 0.05$) decrease in TNF-α concentration as compared to group 2, but significantly ($p< 0.05$) increased when compared to group 3 (24.76±1.87). (Table 4.15; Fig. 4.19).

Nuclear Factor - *Kappa* B (NF-κB)

The NF-κB (pg/mg tissue) in liver homogenate revealed a significant ($p < 0.05$) rise in group 2 (48.65±2.91) as compared to group 1(15.82±4.71).The treatment groups 4 (22.37±3.23), 5(36.52±3.95) and 6 (27.38±3.74) showed a significant ($p < 0.05$) decrease in NF-κB concentration to group 2, but significantly ($p< 0.05$) increased when compared to group 3 (18.46±4.87) (Table 4.16; Fig. 4.20).

Transforming Growth Factor (TGF)-β

The TGF-β (pg/mg tissue) in liver homogenate revealed a significant ($p < 0.05$) rise in group 2 (529.76±4.20) as compared to group 1 (249.81±0.82).The treatment groups 4 (277.66±1.34), 5(313.51±1.93) and 6 (279.61±1.54) showed a significant ($p < 0.05$) decrease in TGF-β concentration to group 2, but significantly ($p < 0.05$) increased

when compared to group 1 (249.81±0.82) and group 3 (241.58±1.05). (Table 4.16; Fig. 4.21)

HISTOPATHOLOGY

Histological examination of liver revealed normal architecture with normal arrangement of hepatic cords and uniform size nucleus in the hepatocytes in group1 (Fig. 4.22) and Hepatocytes showing uniform hepatic cord with uniform size hepatocytes with normal appearance of Kupffer cells in group 3 (Fig. 4.23) where as moderate to severe congestion of portal vein (PV) and central vein (CV), narrowing of hepatic cords, dilated sinusoids and pycnotic nuclei of hepatocytes, mild focal fibrosis of periportal area with mild vasculitis and mild vacoular degeneration of hepatocytes. Severe congestion of central vein and sinusoids and diffuse infilteration of mononuclear cells, severe diffuse vacuolai with moth eaten appearance of cytoplasm and nuclear condensation in group 2 (Fig. 4.24, Fig.4.25, Fig. 4.26 and Fig. 4.27). In group 4 liver showing mild fibrous tissue proliferation around the portal triad and moderate congestion of portal vein, few binucleated hepatocytes and majority of cells showing normal appearance of hepatocytes with normal nuclei and portal triad (Fig. 4.28 and Fig.4.29) and in group 5, normal hepatic cords, with mild congestion of central vein (CV) and sinusoids with mild proliferation of Kupffer cells, severe cellular swelling, mild congestion of central vein and focal infilteration of mononuclear cells (Fig. 4.30 and Fig.4.31) when compared to group 4. The liver showing mild proliferation of Kupffer cells, mild dilatation of sinusoidal and uniform size hepatocytes, near to normal architecture in group 6 (Fig. 4.32 and Fig.4.33).

SPECIAL STAINING

Masson's Trichome Staining (MTS)

The liver sections of group 1 showed normal architecture with specific staining of basement membrane of central vein (Fig. 4.34) and normal architecture with specific staining of basement membrane of central vein in group-3 (Fig. 4.35). In group 2 mild fibrosis in periportal area is seen and moderate proliferation of fibrous tissue around the portal triad along with moderate bile duct proliferation and mild congestion of portal vein (Fig. 4.36 and Fig. 4.37). In group 4 liver sections revealed normal architectural details of liver with central vein and very mild proliferation of fibrous connective tissue around the portal vein (Fig. 4.38 and Fig. 4.39) and group 5 revealed moderate proliferation of fibrous connective tissue around the bile duct (Fig. 4.40 and Fig. 4.41) whereas normal architecture with specific staining of basement membrane of central vein in group 6 (Fig. 4.42 and Fig. 4.43).

Oil Red O staining

The liver sections of group 2 showed mild fatty changes and mild dilated sinusoids, moderate micro and macro vesicular fatty change (Fig. 4.44, Fig. 4.45 and Fig. 4.46). In group 3 liver sections revealed negative for fat stain (Fig. 4.47). In group 4 liver sections revealed very mild positive for fat (Fig. 4.48 and Fig. 4.49) and group 5 showed moderate positive for fat (Fig. 4.50 and Fig. 4.51) whereas group 6 showed mild positive for fat (Fig. 4.52 and Fig. 4.53).

IMMUNOHISTOCHEMICAL ANALYSIS OF BCL-2 IN LIVER

Immunohistochemical analysis was done on tissue sections of mice liver. In group 2 (Fig.4.56 and Fig.4.57) liver sections showed intense cytoplasmic reactivity for Bcl-2 when compared to the group 1 (Fig.4.54 and Fig.4.55). In groups 4 and 6 liver sections showed very mild cytoplasmic immunoreactivity for Bcl-2 (Fig.4.60 and Fig.4.61; Fig.4.64 and Fig.4.65) and in group 5 liver sections showed moderate cytoplasmic immunoreactivity for Bcl-2 (Fig.4.62 and Fig.4.63). The liver sections of group 3 (Fig.4.58 and Fig.4.59) were similar to that of group 1.

GROSS PATHOLOGY OF LIVER

Gross picture of liver in group 2 (Fig.4.67) showed fatty liver, while group 4 (Fig.4.69) showed near to normal with oedema in liver. Group 5 (Fig.4.70) showed mild congestion and oedema in liver, where as groups 1, 3 and 6 showed normal appearance of liver (Fig.4.66, Fig.4.68 and Fig.4.71).

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Table 4.1: Average body weight (g) in different groups of mice

Group	Treatment	0th Week	1st Week	2nd Week	3rd Week	4th Week	5th Week	6th Week
1	Standard diet	28.45±1.68	29.03±2.19 ^{a}	30.10±1.59 ^{a}	30.78±2.07 ^{b}	31.36±1.93 ^{c}	31.80±1.39 ^{c}	32.53±2.38 ^{d}
2	HFD + CCl_4 @ 0.5mg/Kg in olive oil I/P	28.65±5.51	26.86±9.14 ^{c}	28.05±7.21 ^{ab}	29.80±9.21 ^{a}	31.95±6.12 ^{a}	33.05±6.16 ^{a}	36.15±6.14 ^{a}
3	BLF @300mg/Kg p.o	28.45±6.76	28.93±2.08 ^{b}	29.56±2.21 ^{ab}	29.91±2.21 ^{c}	30.55±4.17 ^{d}	31.15±2.07 ^{d}	31.51±1.10 ^{e}
4	HFD +CCl_4 @ 0.5mg/Kg in olive oil I/P + BLF @ 300mg/Kg p.o)	28.60±5.03	27.11±3.29 ^{c}	27.96±2.31 ^{c}	29.08±2.28 ^{d}	30.36±3.24 ^{d}	31.96±3.22 ^{d}	33.11±2.18 ^{c}
5	HFD + CCl_4 @ 0.5mg/Kg in olive oil I/P + BLF @ 100mg/Kg p.o)	28.66±4.33	27.46±4.19 ^{c}	29.01±2.14 ^{b}	31.15±2.22 ^{b}	32.10±3.20 ^{b}	33.26±3.20 ^{b}	34.15±2.12 ^{b}
6	HFD + CCl_4 @ 0.5mg/Kg in olive oil I/P +Standard drug (Simvastatin @ 10mg/Kg p.o)	28.51±4.40	27.38±3.28 ^{c}	29.20±2.14 ^{b}	29.98±3.27 ^{c}	31.83±4.36 ^{bc}	33.10±2.37 ^{bc}	34.13±3.32 ^{b}

Values are Mean + SE (n=6); One way ANOVA with Duncan's post hoc test (SPSS) Means with different alphabets as superscripts differ significantly ($P < 0.05$) among the groups.

Fig 4.1: Average body weight (g) in different groups of mice

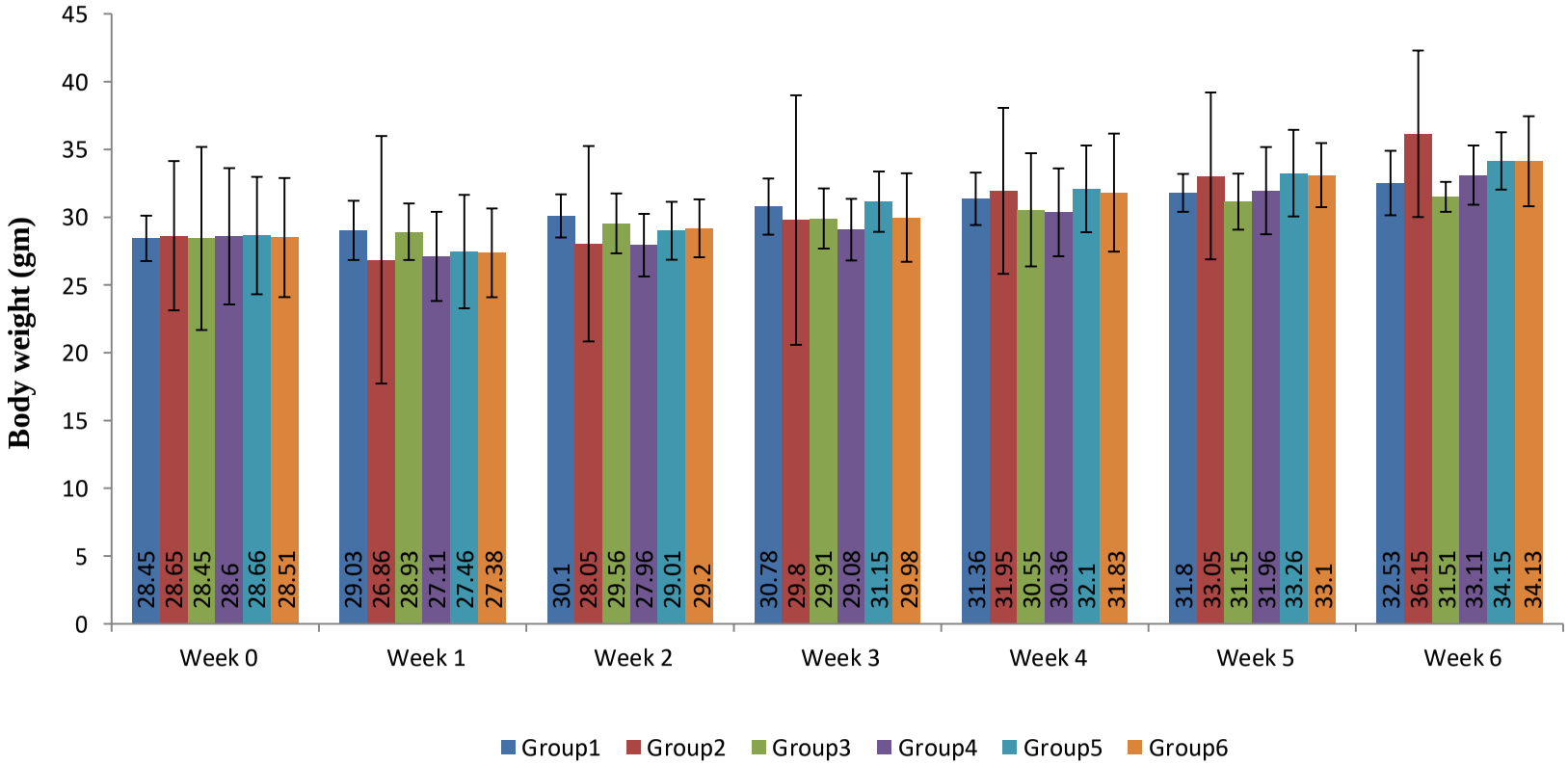


Table 4.2: Average liver weight (g) & Relative liver weight (%) in different groups of mice

Group	Treatment	Average liver weight (g)	Relative liver weight (%)
1	Standard diet	1.41±0.41^{c}	4.33±0.05^{c}
2	HFD + CCl_4 (0.5mg/Kg b.wt in olive oil I.P	2.12±1.31^{a}	5.86±0.29^{a}
3	BLF (300mg/Kg b.wt p.o	1.33±0.36^{d}	4.22±0.08^{d}
4	HFD +CCl_4 (0.5mg/Kg b.wt in olive oil I.P + BLF (300mg/Kg b.wt p.o)	1.47±0.51^{c}	4.43±0.06^{c}
5	HFD + CCl_4 (0.5mg/Kg b.wt in olive oil I.P + BLF (100mg/Kg b.wt p.o)	1.56±0.93^{b}	4.56±0.04^{b}
6	HFD + CCl_4 (0.5mg/Kg b.wt in olive oil I.P +Standard drug (Simvastatin (@10mg/Kg b.wt p.o)	1.54±0.86^{b}	4.51±0.04^{b}

Values are Mean ± SE (n=6); One way ANOVA with Duncan's post hoc test (SPSS) Means with different alphabets as superscripts differ significantly ($p < 0.05$) among the groups

Table 4.3: Total cholesterol concentration (mg/dL) in different groups of mice

Group	Treatment	2nd Week	4th Week	6th Week
1	Standard diet	91.20±2.82^{e}	92.90±1.26^{d}	94.50±1.70^{e}
2	HFD + CCl_4 (0.5mg/Kg b.wt in olive oil I.P	163.13±4.36^{a}	182.35±6.26^{a}	203.66±8.99^{a}
3	BLF (300mg/Kg b.wt p.o	91.23±6.64^{e}	89.03±2.61^{e}	86.78±3.50^{f}
4	HFD +CCl_4 (0.5mg/Kg b.wt in olive oil I.P + BLF (300mg/Kg b.wt p.o)	147.85±5.23^{c}	123.95±2.69^{c}	116.08±2.78^{c}
5	HFD + CCl_4 (0.5mg/Kg b.wt in olive oil I.P + BLF (100mg/Kg b.wt p.o)	152.11±4.77^{b}	136.23±2.71^{b}	123.03±2.89^{b}
6	HFD + CCl_4 (0.5mg/Kg b.wt in olive oil I.P +Standard drug (Simvastatin @ 10mg/Kg b.wt p.o)	132.05±3.95^{d}	124.01±3.52^{c}	98.31±3.06^{d}

Values are Mean ± SE (n=6); One way ANOVA with Duncan's post hoc test (SPSS) Means with different alphabets as superscripts differ significantly ($p < 0.05$) among the groups

Table 4.4: Triglycerides concentration (mg/dL) in different groups of mice

Group	Treatment	2nd Week	4th Week	6th Week
1	Standard diet	57.08±3.04^{e}	62.03±3.29^{e}	66.03±2.37^{e}
2	HFD + CCl_4 (0.5mg/Kg b.wt in olive oil I.P	92.03±9.90^{a}	116.30±13.55^{a}	121.95±11.84^{a}
3	BLF (300mg/Kg b.wt p.o	55.95±7.49^{f}	52.63±6.38^{f}	49.03±5.99^{f}
4	HFD +CCl_4 (0.5mg/Kg b.wt in olive oil I.P + BLF (300mg/Kg b.wt p.o)	82.03±7.70^{c}	74.95±9.08^{c}	71.06±7.84^{c}
5	HFD + CCl_4 (0.5mg/Kg b.wt in olive oil I.P + BLF (100mg/Kg b.wt p.o)	84.03±6.59^{b}	77.95±7.16^{b}	73.91±6.37^{b}
6	HFD + CCl_4 (0.5mg/Kg b.wt in olive oil I.P +Standard drug (Simvastatin @10mg/Kg b.wt p.o)	81.10±6.79^{d}	74.35±8.79^{d}	68.00±6.85^{d}

Values are Mean ± SE (n=6); One way ANOVA with Duncan's post hoc test (SPSS) Means with different alphabets as superscripts differ significantly ($p < 0.05$) among the groups

Table 4.5: HDL Cholesterol concentration (mg/dL) in different groups of mice

Group	Treatment	2nd Week	4th Week	6th Week
1	Standard diet	49.36±1.25^{b}	50.23±1.19^{b}	52.15±1.44^{c}
2	HFD + CCl_4 (0.5mg/Kg b.wt in olive oil I.P	30.76±0.55^{f}	23.55±0.70^{f}	16.80±0.67^{e}
3	BLF (300mg/Kg b.wt p.o	50.40±1.02^{a}	56.40±1.21^{a}	59.81±1.45^{a}
4	HFD +CCl_4 (0.5mg/Kg b.wt in olive oil I.P + BLF (300mg/Kg b.wt p.o)	42.21±1.04^{d}	48.96±2.05^{d}	52.05±1.50^{c}
5	HFD + CCl_4 (0.5mg/Kg b.wt in olive oil I.P + BLF (100mg/Kg b.wt p.o)	39.15±0.92^{e}	46.03±1.24^{e}	49.21±1.80^{d}
6	HFD + CCl_4 (0.5mg/Kg b.wt in olive oil I.P +Standard drug (Simvastatin @ 10mg/Kg b.wt p.o)	43.18±0.97^{c}	51.11±1.02^{c}	55.25±1.72^{b}

Values are Mean ± SE (n=6); One way ANOVA with Duncan's post hoc test (SPSS) Means with different alphabets as superscripts differ significantly ($p < 0.05$) among the groups

Table 4.6: Glucose concentration (mg/dL) in different groups of mice

Group	Treatment	2nd Week	4th Week	6th Week
1	Standard diet	241.66± 1.37^{e}	246.16± 1.98^{d}	250.50± 2.47^{d}
2	HFD + CCl_4 (0.5mg/Kg b.wt in olive oil I.P	287.66±8.81^{a}	299.16±6.49^{a}	310.33±5.79^{a}
3	BLF (300mg/Kg b.wt p.o	239.16± 1.33^{e}	235.5± 1.68^{e}	231.83± 2.15^{e}
4	HFD +CCl_4 (0.5mg/Kg b.wt in olive oil I.P + BLF (300mg/Kg b.wt p.o)	264.83± 3.63^{d}	260.00± 3.94^{c}	256.50± 3.14^{cd}
5	HFD + CCl_4 (0.5mg/Kg b.wt in olive oil I.P + BLF (100mg/Kg b.wt p.o)	271.50±3.32^{c}	262.33±3.84^{c}	259.83±3.74^{c}
6	HFD + CCl_4 (0.5mg/Kg b.wt in olive oil I.P +Standard drug (Simvastatin @ 10mg/Kg b.wt p.o)	282.33± 3.50^{b}	276.16± 4.26^{b}	272.33±3.80^{b}

Values are Mean ± SE (n=6); One way ANOVA with Duncan's post hoc test (SPSS) Means with different alphabets as superscripts differ significantly ($p < 0.05$) among the groups

Table 4.7: Serum alanine transaminase (ALT) levels (IU/L) in different groups of mice

Group	Treatment	2nd Week	4th Week	6th Week
1	Standard diet	40.68±1.41^{e}	43.10±1.23^{e}	45.23±1.20^{e}
2	HFD + CCl_4 (0.5mg/Kg b.wt in olive oil I.P	204.16±1.79^{a}	228.18±1.44^{a}	244.18±1.13^{a}
3	BLF (300mg/Kg b.wt p.o	39.31±1.40^{f}	37.06±1.24^{f}	34.03±1.46^{f}
4	HFD +CCl_4 (0.5mg/Kg b.wt in olive oil I.P + BLF (300mg/Kg b.wt p.o)	129.16±1.37^{d}	101.06±1.33^{d}	84.13±1.10^{d}
5	HFD + CCl_4 (0.5mg/Kg b.wt in olive oil I.P + BLF (100mg/Kg b.wt p.o)	131.10±1.15^{c}	106.20±1.28^{c}	96.21±1.19^{c}
6	HFD + CCl_4 (0.5mg/Kg b.wt in olive oil I.P +Standard drug (Simvastatin @ 10mg/Kg b.wt p.o)	132.43±1.29^{b}	127.06±1.20^{b}	113.03±1.27^{b}

Values are Mean ± SE (n=6); One way ANOVA with Duncan's post hoc test (SPSS) Means with different alphabets as superscripts differ significantly ($p < 0.05$) among the groups

Table 4.8: Serum Aspertate transaminase (AST) levels (IU/L) in different groups of mice

Group	Treatment	2nd Week	4th Week	6th Week
1	Standard diet	121.15±0.47 ^{e}	123.13±1.35 ^{e}	128.11±2.11 ^{e}
2	HFD + CCl_4 (0.5mg/Kg b.wt in olive oil I.P	213.93±1.21 ^{a}	237.88±1.74 ^{a}	264.93±1.23 ^{a}
3	BLF (300mg/Kg b.wt p.o	120.98±1.65 ^{f}	119.15±1.65 ^{f}	117.06±1.48 ^{f}
4	HFD +CCl_4 (0.5mg/Kg b.wt in olive oil I.P + BLF (300mg/Kg b.wt p.o)	152.11±1.28 ^{d}	142.28±1.28 ^{d}	133.90±1.11 ^{d}
5	HFD + CCl_4 (0.5mg/Kg b.wt in olive oil I.P + BLF (100mg/Kg b.wt p.o)	156.20±1.24 ^{b}	148.08±1.24 ^{b}	139.13±1.23 ^{b}
6	HFD + CCl_4 (0.5mg/Kg b.wt in olive oil I.P +Standard drug (Simvastatin @ 10mg/Kg b.wt p.o)	154.15±1.49 ^{c}	143.96±1.49 ^{c}	136.10±1.39 ^{c}

Values are Mean ± SE (n=6); One way ANOVA with Duncan's post hoc test (SPSS) Means with different alphabets as superscripts differ significantly ($p < 0.05$) among the groups

Table 4.9: Serum total protein concentration (g/dL) in different groups of mice

Group	Treatment	2nd Week	4th Week	6th Week
1	Standard diet	7.57±0.07^{b}	7.56±0.08^{b}	7.55±0.09^{d}
2	HFD + CCl_4 (0.5mg/Kg b.wt in olive oil I.P	4.59±0..06^{f}	4.47±0.05^{f}	4.43±0.05^{f}
3	BLF (300mg/Kg b.wt p.o	7.94±0.03^{a}	8.02±0.12^{a}	8.12±0.09^{a}
4	HFD +CCl_4 (0.5mg/Kg b.wt in olive oil I.P + BLF (300mg/Kg b.wt p.o)	6.58±0.02^{d}	7.10±0.04^{c}	7.40±0.07^{b}
5	HFD + CCl_4 (0.5mg/Kg b.wt in olive oil I.P + BLF (100mg/Kg b.wt p.o)	5.56±0.02^{e}	6.67±0.04^{e}	6.91±0.08^{e}
6	HFD + CCl_4 (0.5mg/Kg b.wt in olive oil I.P +Standard drug (Simvastatin @ 10mg/Kg b.wt p.o)	6.28±0.03^{c}	6.89±0.04^{d}	7.02±0.09^{c}

Values are Mean ± SE (n=6); One way ANOVA with Duncan's post hoc test (SPSS) Means with different alphabets as superscripts differ significantly ($p < 0.05$) among the groups

Table 4.10: Serum total bilirubin concentration (mg/dL) in different groups of mice

Group	Treatment	2nd Week	4th Week	6th Week
1	Standard diet	0.13 ± 0.01^{d}	0.15 ± 0.03^{d}	0.15 ± 0.02^{d}
2	HFD + CCl_4 (0.5mg/Kg b.wt in olive oil I.P	0.24 ± 0.01^{a}	0.31 ± 0.01^{a}	0.38 ± 0.01^{a}
3	BLF (300mg/Kg b.wt p.o	0.11 ± 0.01^{e}	0.12 ± 0.01^{e}	0.13 ± 0.01^{e}
4	HFD +CCl_4 (0.5mg/Kg b.wt in olive oil I.P + BLF (300mg/Kg b.wt p.o)	0.19 ± 0.01^{c}	0.18 ± 0.01^{c}	0.17 ± 0.01^{c}
5	HFD + CCl_4 (0.5mg/Kg b.wt in olive oil I.P + BLF (100mg/Kg b.wt p.o)	0.21 ± 0.01^{b}	0.20 ± 0.01^{b}	0.19 ± 0.01^{b}
6	HFD + CCl_4 (0.5mg/Kg b.wt in olive oil I.P +Standard drug (Simvastatin @ 10mg/Kg b.wt p.o)	0.21 ± 0.01^{b}	0.20 ± 0.01^{b}	0.19 ± 0.01^{b}

Values are Mean ± SE (n=6); One way ANOVA with Duncan's post hoc test (SPSS) Means with different alphabets as superscripts differ significantly ($p < 0.05$) among the groups

Table 4.11: Serum *gamma*-glutamyl transferase (GGT) levels (U/L) in different groups of mice

Group	Treatment	2nd Week	4th Week	6th Week
1	Standard diet	5.64 ± 0.12^{e}	5.88 ± 0.40^{e}	5.92 ± 0.21^{d}
2	HFD + CCl_4 (0.5mg/Kg b.wt in olive oil I.P	16.67 ± 0.8^{a}	19.66 ± 0.20^{a}	21.80 ± 0.19^{a}
3	BLF (300mg/Kg b.wt p.o	5.08 ± 1.15^{f}	5.15 ± 0.40^{f}	5.42 ± 0.30^{f}
4	HFD +CCl_4 (0.5mg/Kg b.wt in olive oil I.P + BLF (300mg/Kg b.wt p.o)	12.62 ± 1.30^{c}	9.46 ± 0.60^{b}	6.53 ± 0.51^{c}
5	HFD + CCl_4 (0.5mg/Kg b.wt in olive oil I.P + BLF (100mg/Kg b.wt p.o)	13.62 ± 0.80^{d}	10.85 ± 0.50^{d}	8.94 ± 0.43^{e}
6	HFD + CCl_4 (0.5mg/Kg b.wt in olive oil I.P +Standard drug (Simvastatin @10mg/Kg b.wt p.o)	12.66 ± 1.70^{b}	9.87 ± 0.98^{c}	6.61 ± 0.98^{b}

Values are Mean ± SE (n=6); One way ANOVA with Duncan's post hoc test (SPSS) Means with different alphabets as superscripts differ significantly ($p < 0.05$) among the groups

Table 4.12: GSH concentration in liver (nmoles /mg protein) & TBARS concentration in liver (n M of MDA/mg protein) of different groups of mice

Group	Treatment	GSH concentration in liver (nmoles /mg protein)	TBARS concentration in liver (n M of MDA/mg protein)
1	Standard diet	24.73 ± 1.16^{a}	2.19 ± 0.08^{d}
2	HFD + CCl_4 (0.5mg/Kg b.wt in olive oil I.P	14.98 ± 0.44^{e}	3.93 ± 0.04^{a}
3	BLF (300mg/Kg b.wt p.o	24.81 ± 0.54^{a}	2.09 ± 0.10^{e}
4	HFD +CCl_4 (0.5mg/Kg b.wt in olive oil I.P + BLF (300mg/Kg b.wt p.o)	23.15 ± 0.67^{b}	2.48 ± 0.04^{c}
5	HFD + CCl_4 (0.5mg/Kg b.wt in olive oil I.P + BLF (100mg/Kg b.wt p.o)	21.24 ± 0.39^{d}	2.95 ± 0.06^{b}
6	HFD + CCl_4 (0.5mg/Kg b.wt in olive oil I.P +Standard drug Simvastatin @ 10mg/Kg b.wt p.o)	22.91 ± 0.31^{c}	2.67 ± 0.02^{c}

Values are Mean ± SE (n=6); One way ANOVA with Duncan's post hoc test (SPSS) Means with different alphabets as superscripts differ significantly ($p < 0.05$) among the groups

Table 4.13: Catalase activity in liver (U /mg protein) & Superoxide dismutase (SOD) activity in liver (U/mg protein) of different groups of mice

Group	Treatment	Catalase activity in liver (U /mg protein)	Superoxide dismutase (SOD) activity in liver (U/mg protein)
1	Standard diet	71.75 ± 4.80^{b}	6.99 ± 0.29^{a}
2	HFD + CCl_4 (0.5mg/Kg b.wt in olive oil I.P	43.75 ± 4.10^{f}	3.47 ± 0.11^{f}
3	BLF (300mg/Kg b.wt p.o	72.69 ± 5.92^{a}	6.26 ± 0.25^{b}
4	HFD +CCl_4 (0.5mg/Kg b.wt in olive oil I.P + BLF (300mg/Kg b.wt p.o)	68.91 ± 3.23^{c}	5.87 ± 0.05^{c}
5	HFD + CCl_4 (0.5mg/Kg b.wt in olive oil I.P + BLF (100mg/Kg b.wt p.o)	65.78 ± 3.00^{e}	5.12 ± 0.02^{e}
6	HFD + CCl_4 (0.5mg/Kg b.wt in olive oil I.P +Standard drug (Simvastatin @ 10mg/Kg b.wt p.o)	67.66 ± 3.43^{d}	5.74 ± 0.05^{d}

Values are Mean ± SE (n=6); One way ANOVA with Duncan's post hoc test (SPSS) Means with different alphabets as superscripts differ significantly ($p < 0.05$) among the groups

Table 4.14: Nitric oxide concentration in liver (µM of nitrite/mg tissue) of different groups of mice

Group	Treatment	6th Week
1	Standard diet	2.75±0.13^{d}
2	HFD + CCl_4 (0.5mg/Kg b.wt in olive oil I.P	8.46±0.72^{a}
3	BLF (300mg/Kg b.wt p.o	2.58±0.18^{e}
4	HFD +CCl_4 (0.5mg/Kg b.wt in olive oil I.P + BLF (300mg/Kg b.wt p.o)	3.28±0.21^{c}
5	HFD + CCl_4 (0.5mg/Kg b.wt in olive oil I.P + BLF (100mg/Kg b.wt p.o)	4.17±0.16^{b}
6	HFD + CCl_4 (0.5mg/Kg b.wt in olive oil I.P +Standard drug (Simvastatin @ 10mg/Kg b.wt p.o)	3.18±0.17^{c}

Values are Mean ± SE (n=6); One way ANOVA with Duncan's post hoc test (SPSS) Means with different alphabets as superscripts differ significantly ($p < 0.05$) among the groups

Table 4.15 : IL-10 levels in liver (pg/mg tissue) & TNF- α levels in liver (pg/mg tissue) activity in liver (U/mg protein) of different groups of mice

Group	Treatment	IL-10 levels in liver (pg/mg tissue)	TNF- α levels in liver (pg/mg tissue)
1	Standard diet	71.61±0.42^{a}	26.62±2.21^{e}
2	HFD + CCl_4 (0.5mg/Kg b.wt in olive oil I.P	55.45±0.30^{e}	52.65±3.45^{a}
3	BLF (300mg/Kg b.wt p.o	69.68±0.33^{b}	24.76±1.87^{f}
4	HFD +CCl_4 (0.5mg/Kg b.wt in olive oil I.P + BLF (300mg/Kg b.wt p.o)	65.54±0.37^{c}	28.67±2.23^{c}
5	HFD + CCl_4 (0.5mg/Kg b.wt in olive oil I.P + BLF (100mg/Kg b.wt p.o)	61.62±0.36^{d}	36.87±2.05^{b}
6	HFD + CCl_4 (0.5mg/Kg b.wt in olive oil I.P +Standard drug (Simvastatin (@10mg/Kg b.wt p.o)	64.69±0.32^{d}	27.73±2.34^{d}

Values are Mean ± SE (n=6); One way ANOVA with Duncan's post hoc test (SPSS) Means with different alphabets as superscripts differ significantly ($p < 0.05$) among the groups

Table 4.16: NF-κB levels in liver (pg/mg tissue) & TGF-β levels in liver (pg/mg tissue) of different groups of mice

Group	Treatment	NF-κB levels in liver (pg/mg tissue)	TGF-β levels in liver (pg/mg tissue)
1	Standard diet	15.82 ± 4.71^{f}	249.81 ± 0.82^{e}
2	HFD + CCl_4 (0.5mg/Kg b.wt in olive oil I.P	48.65 ± 2.91^{a}	529.76 ± 4.20^{a}
3	BLF (300mg/Kg b.wt p.o	18.46 ± 4.87^{e}	241.58 ± 1.05^{f}
4	HFD +CCl_4 (0.5mg/Kg b.wt in olive oil I.P + BLF (300mg/Kg b.wt p.o)	22.37 ± 3.23^{d}	277.66 ± 1.34^{d}
5	HFD + CCl_4 (0.5mg/Kg b.wt in olive oil I.P + BLF (100mg/Kg b.wt p.o)	36.52 ± 3.95^{b}	313.51 ± 1.93^{b}
6	HFD + CCl_4 (0.5mg/Kg b.wt in olive oil I.P +Standard drug (Simvastatin @ 10mg/Kg b.wt p.o)	27.38 ± 3.74^{c}	279.61 ± 1.54^{c}

Values are Mean ± SE (n=6); One way ANOVA with Duncan's post hoc test (SPSS) Means with different alphabets as superscripts differ significantly ($p < 0.05$) among the groups

Fig. 4.2: Average liver weight (g) of different groups of mice

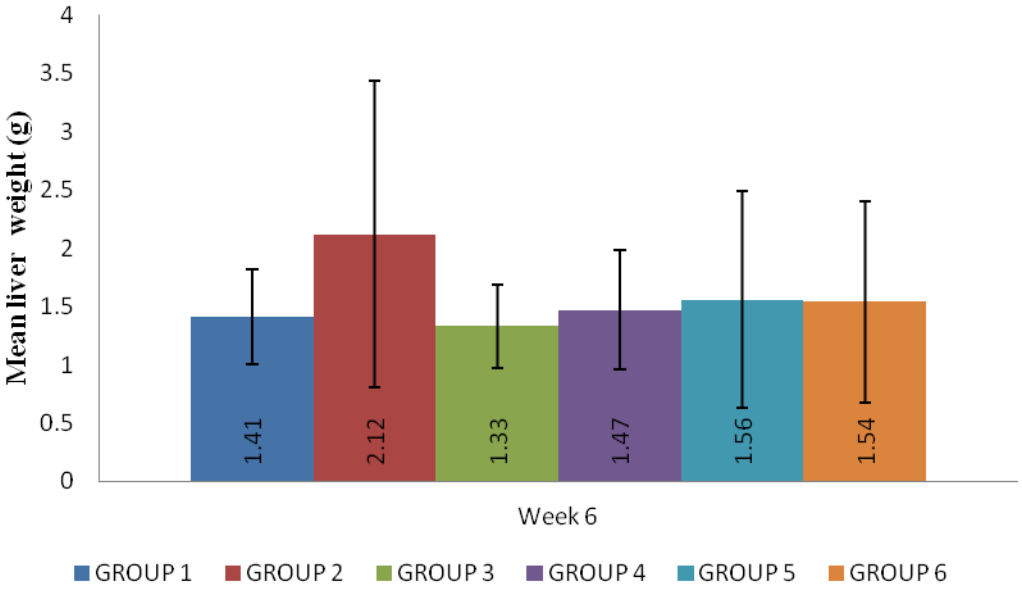
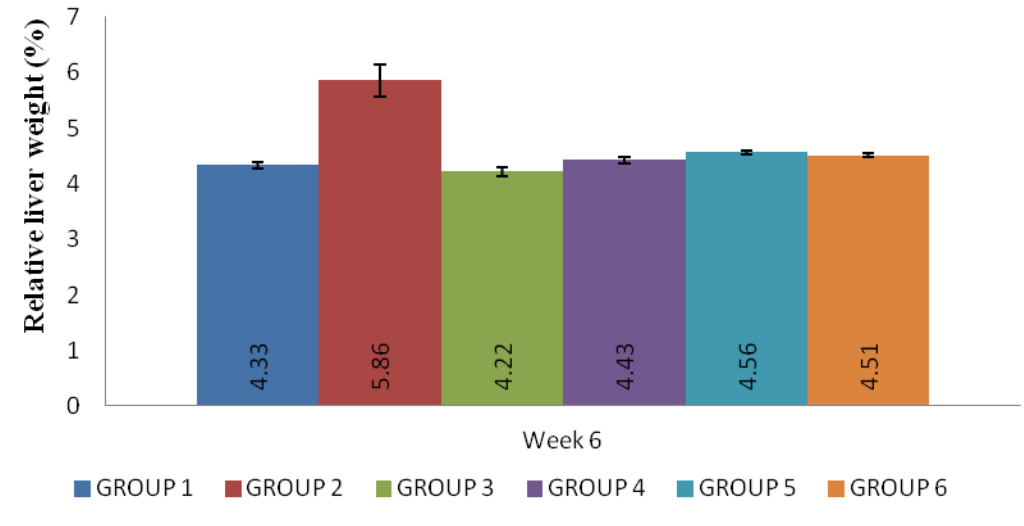


Fig. 4.3: Relative Liver weight (%) of different groups of mice



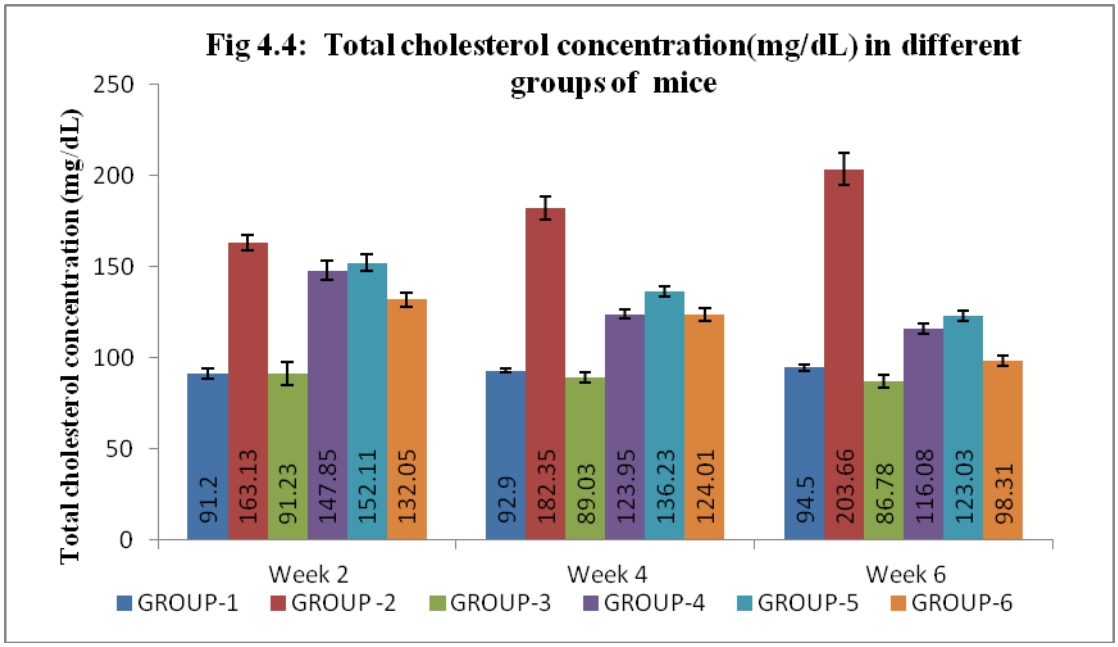


Fig 4.4: Total cholesterol concentration(mg/dL) in different groups of mice
Total cholesterol concentration (mg/dL)
250
200
150
100
50
0
91.2
163.13
91.23
147.85
152.11
132.05
92.9
182.35
89.03
123.95
136.23
124.01
94.5
203.66
86.78
116.08
123.03
98.31
Week 2
Week 4
Week 6
GROUP-1
GROUP -2
GROUP-3
GROUP-4
GROUP-5
GROUP-6

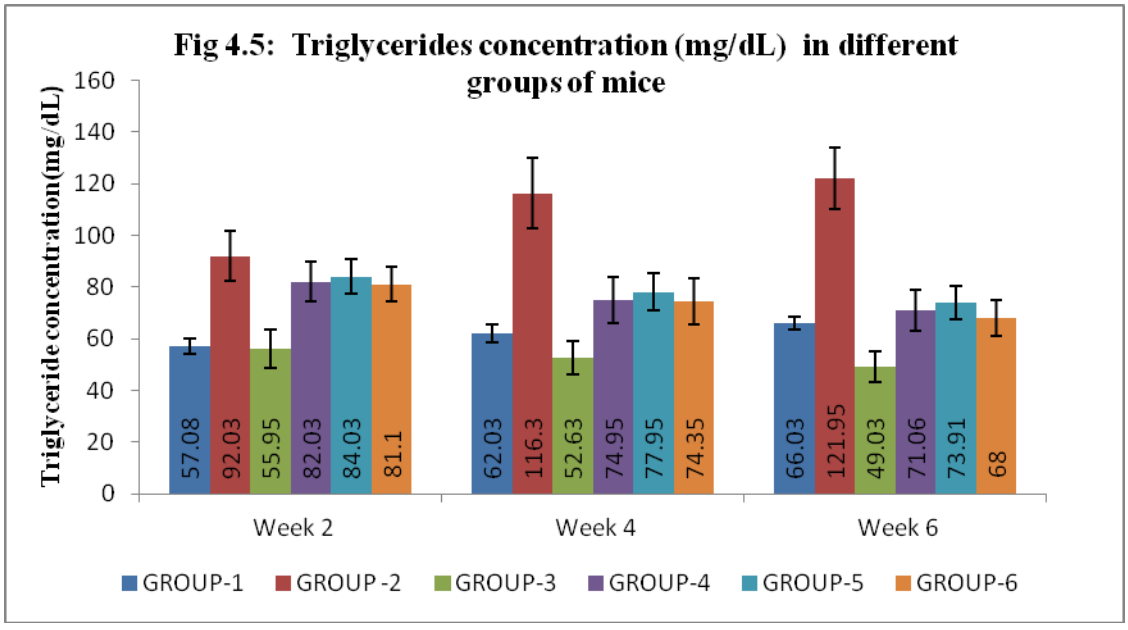


Fig 4.5: Triglycerides concentration (mg/dL) in different groups of mice
Triglyceride concentration(mg/dL)
160
140
120
100
80
60
40
20
0
57.08
92.03
55.95
82.03
84.03
81.1
62.03
116.3
52.63
74.95
77.95
74.35
66.03
121.95
49.03
71.06
73.91
68
Week 2
Week 4
Week 6
GROUP-1
GROUP -2
GROUP-3
GROUP-4
GROUP-5
GROUP-6

Fig 4.6: HDL cholesterol concentration (mg/dL) in different groups of mice

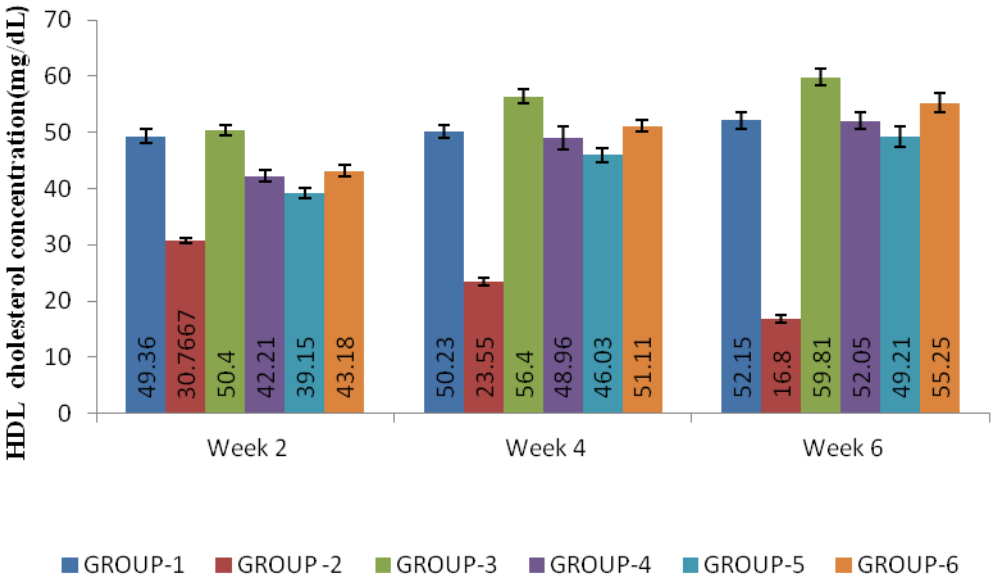


Fig 4.7: Glucose concentration (mg/dL) in different groups of mice

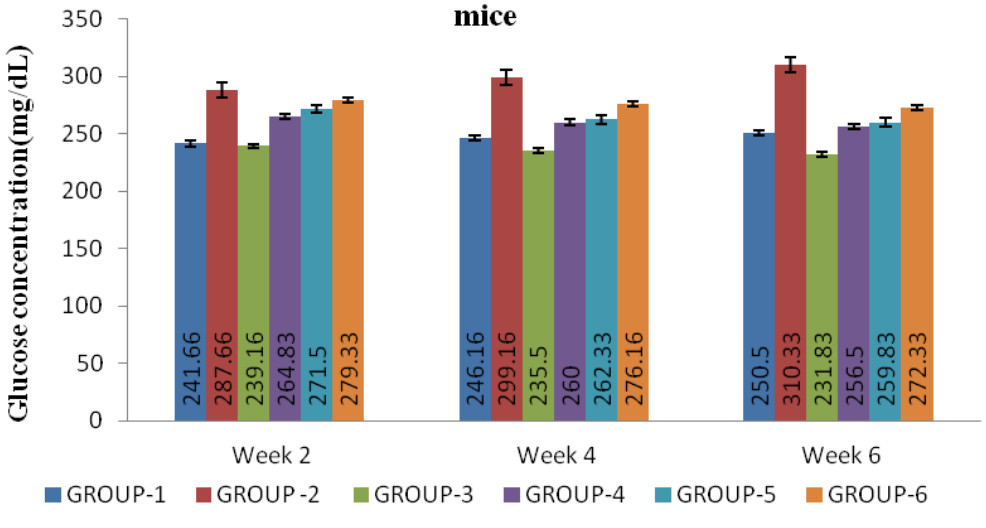


Fig 4.8: Serum alanine transaminase (ALT) levels (IU/L) in different groups of mice

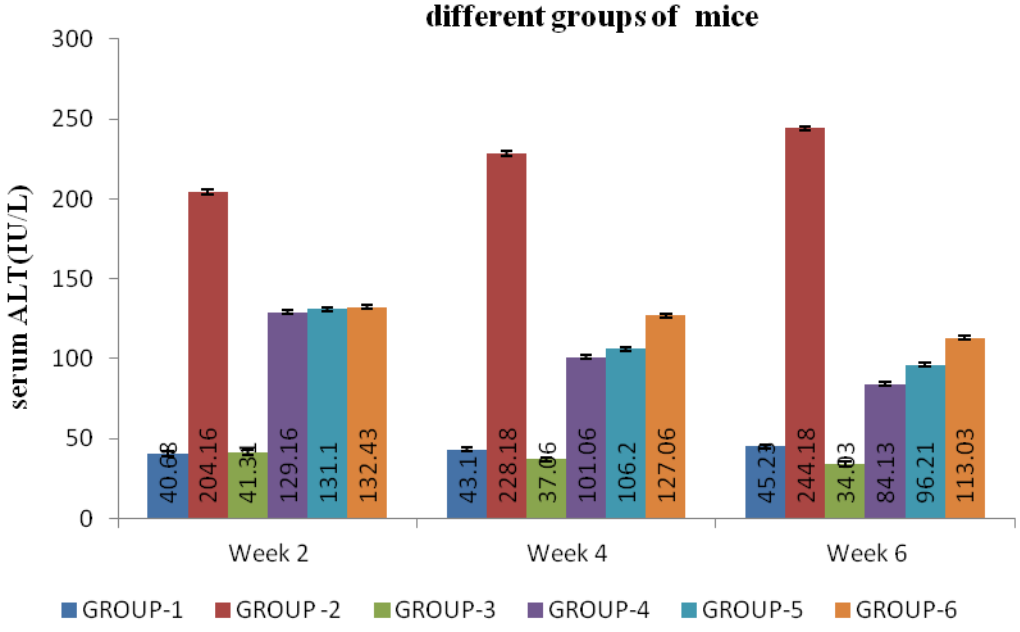


Fig 4.9: Serum Aspertate transaminase (AST) levels (IU/L) in different groups of mice

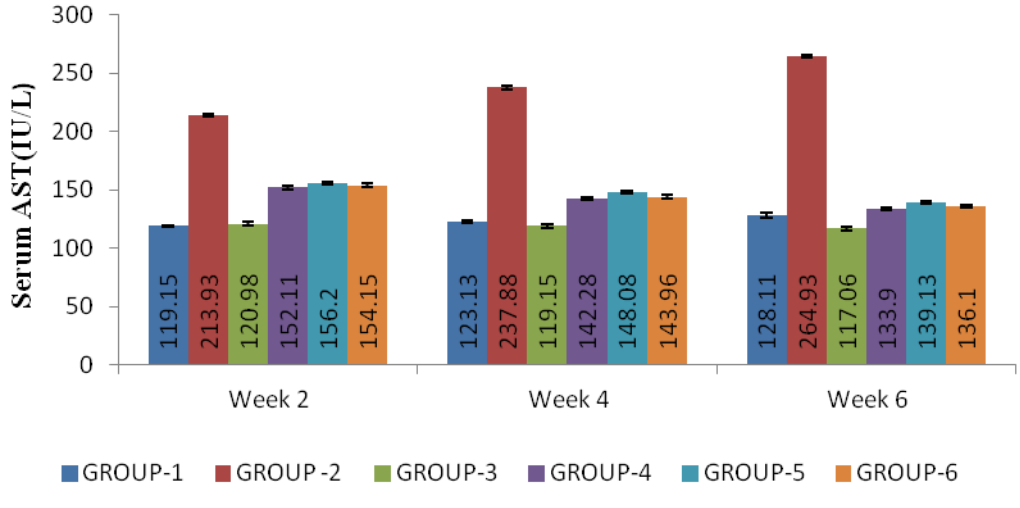


Fig 4.10: Serum total protein concentration (g/dL) in different groups of mice

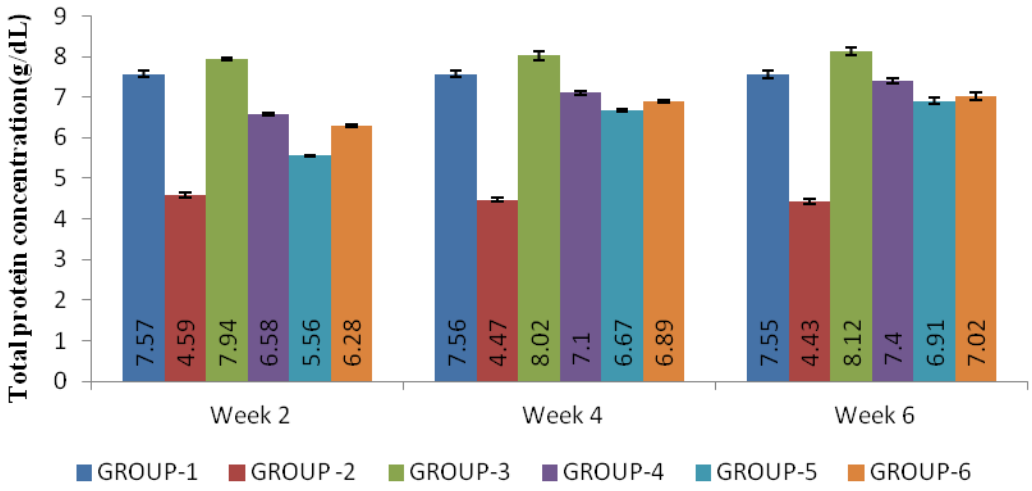


Fig 4.11: Serum total bilirubin concentration (mg/dL) in different groups of mice

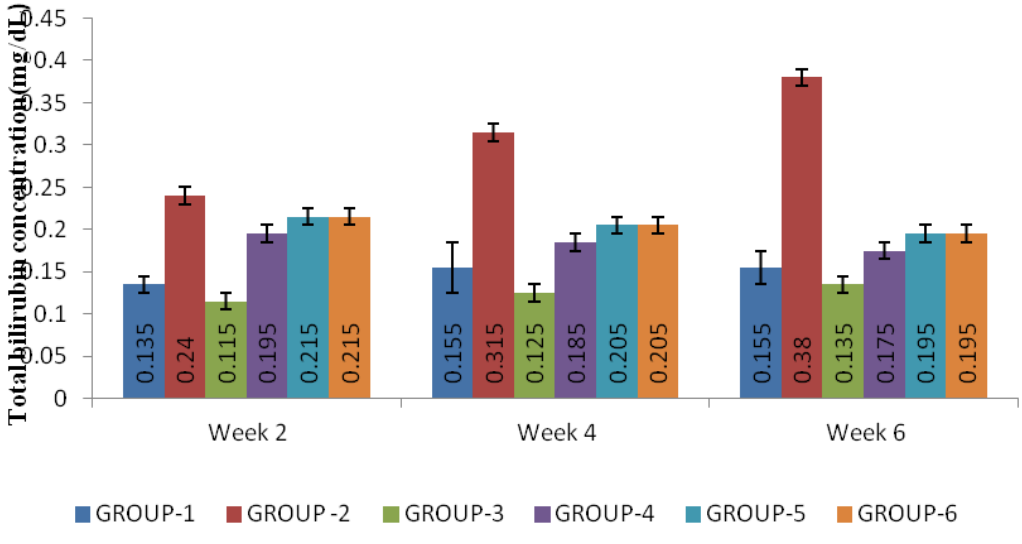


Fig 4.12: Serum gamma-glutamyl transferase (GGT) levels (U/L) in different groups of mice

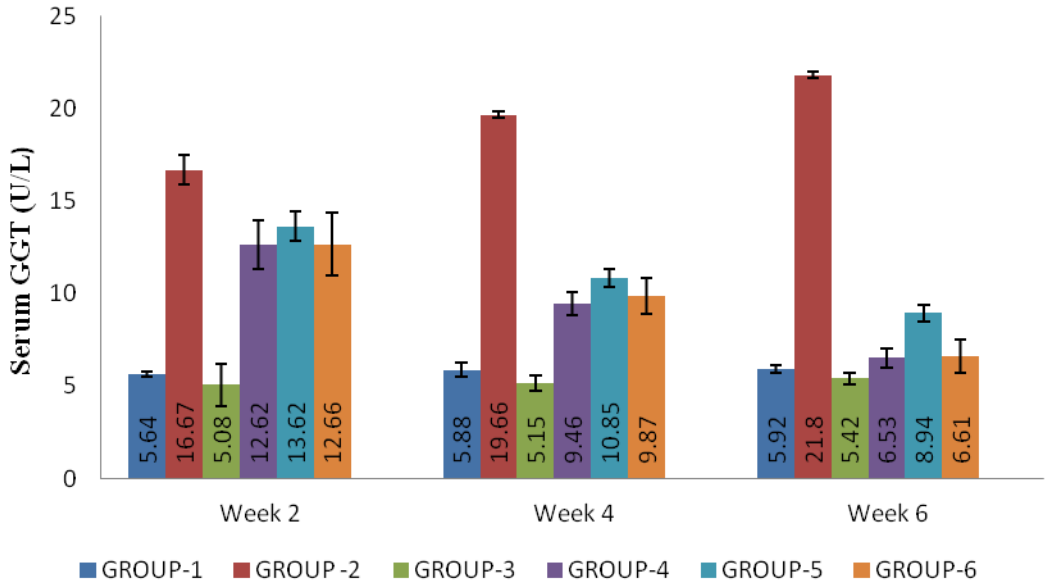


Fig 4.13: GSH concentration in liver (nmol/mg protein) of different groups of mice

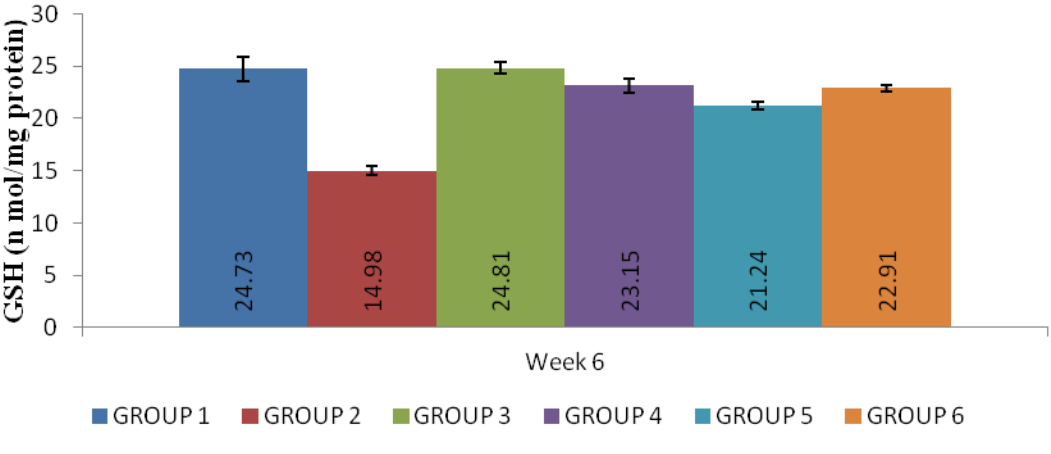


Fig 4.14: Catalase activity in liver (U /mg protein) of different groups of mice

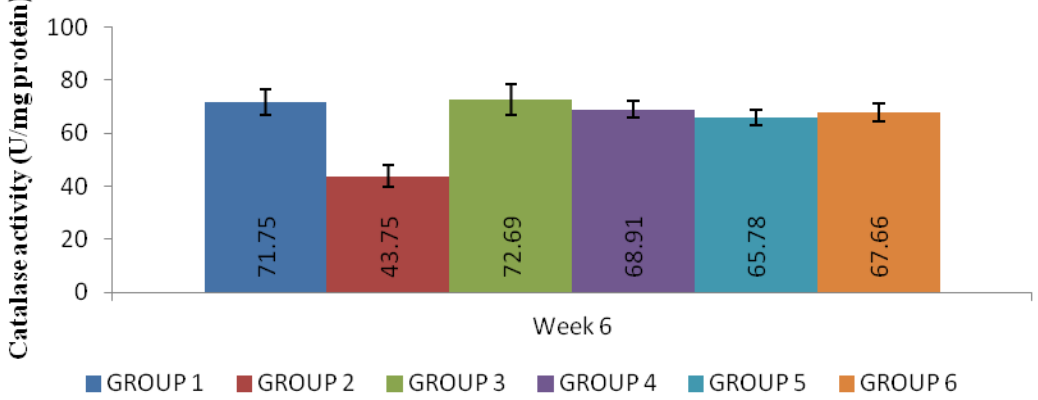


Fig 4.15: Superoxide dismutase (SOD) activity in liver (U/mg protein) of different groups of mice

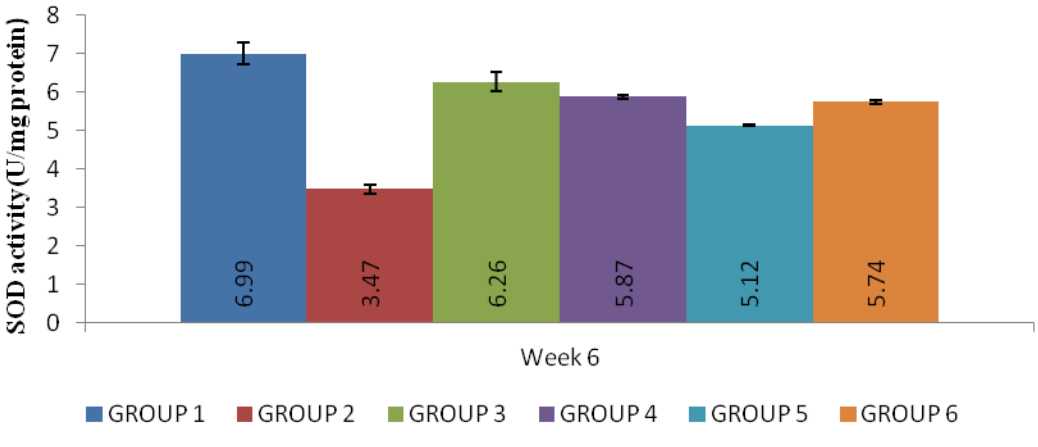


Fig 4.16: TBARS concentration in liver (n M of MDA/mg protein) of different groups of mice

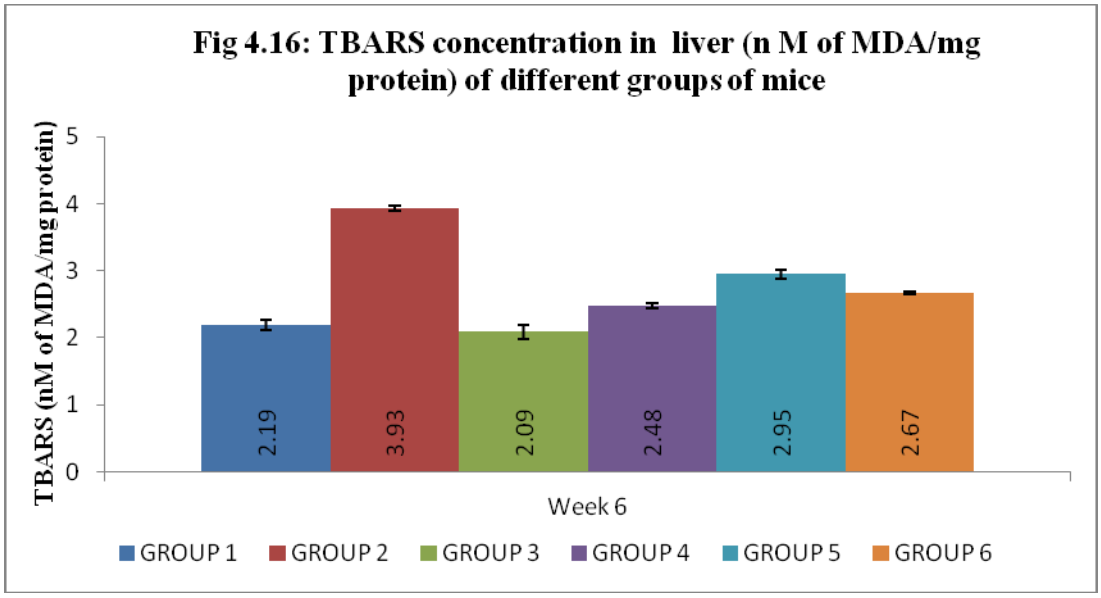


Fig 4.17: Nitric oxide concentration in liver (µM of nitrite/mg tissue) of different groups of mice

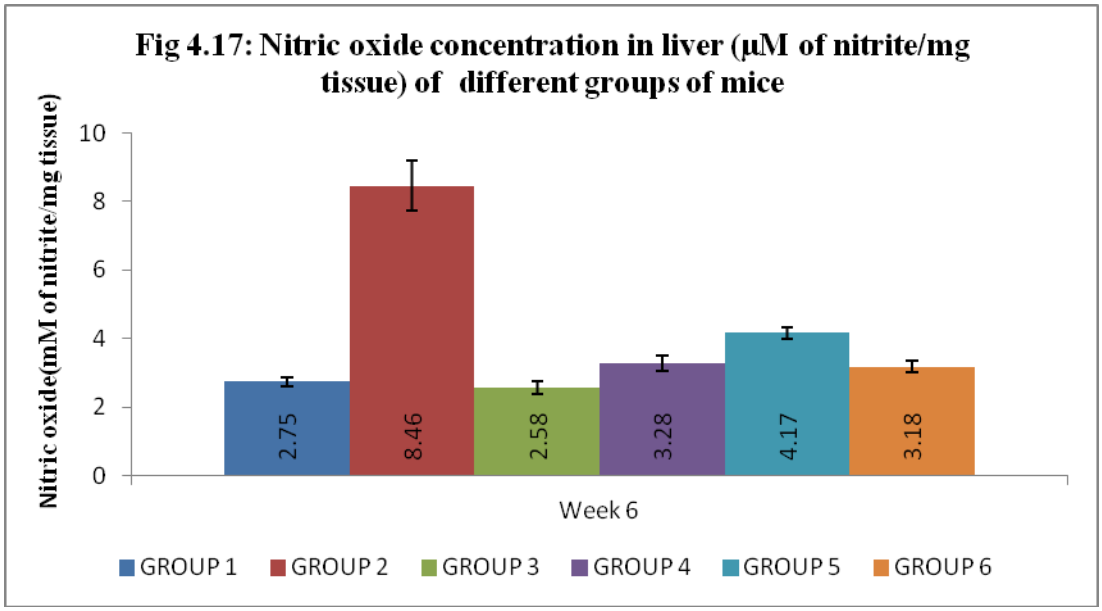


Fig 4.18: IL-10 levels in liver (pg/mg tissue) of different groups of mice

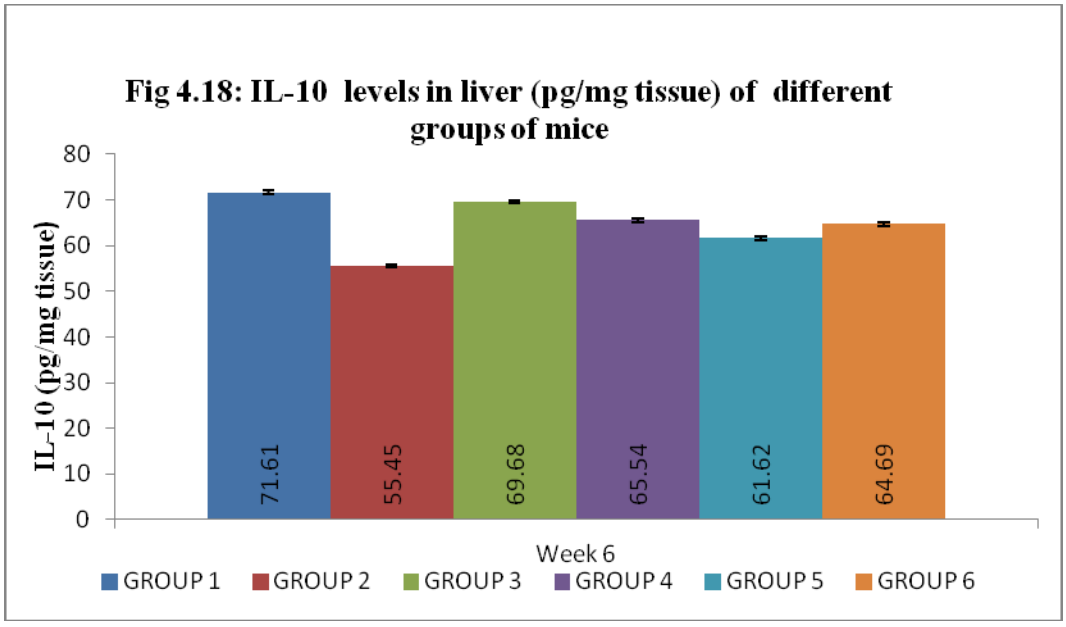


Fig 4.19: TNF- α levels in liver (pg/mg tissue) of different groups of mice

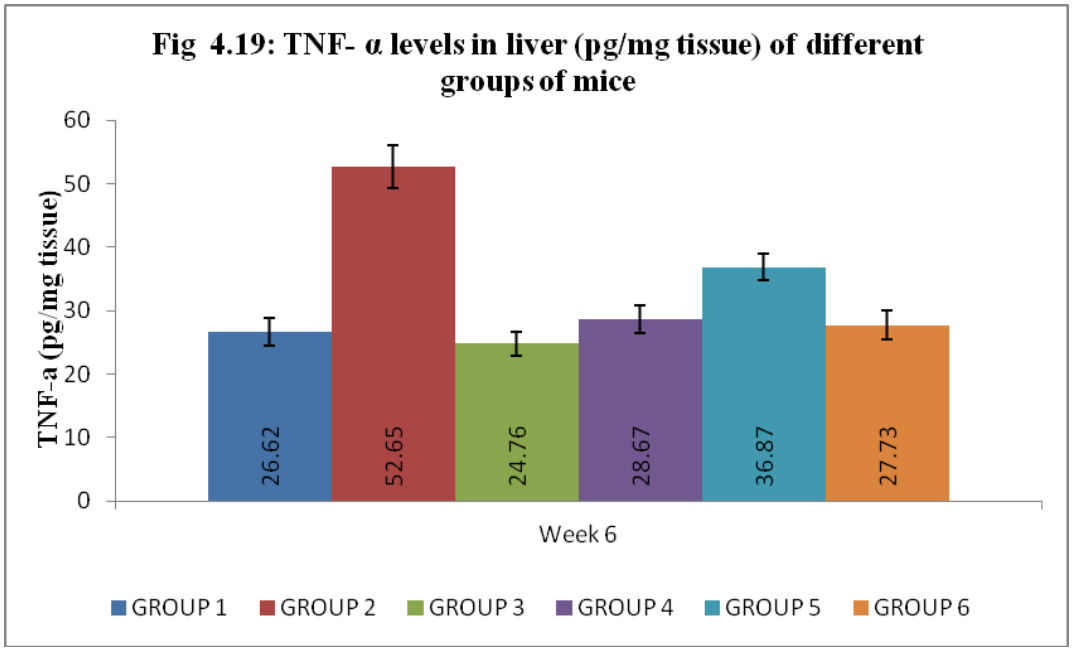


Fig 4.20: NF-κB levels in liver (pg/mg tissue) of different groups of mice

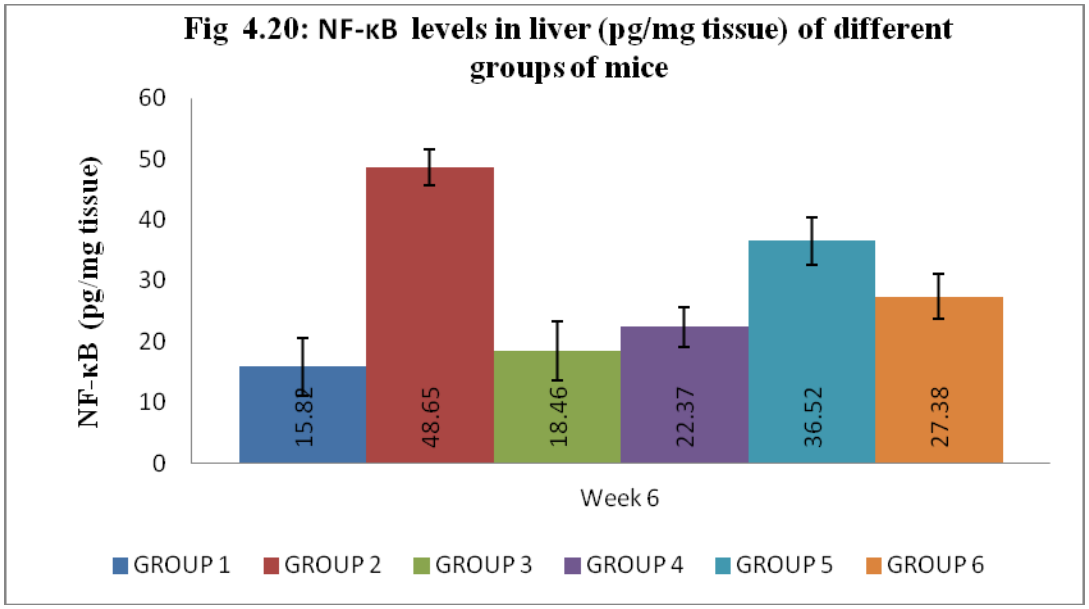
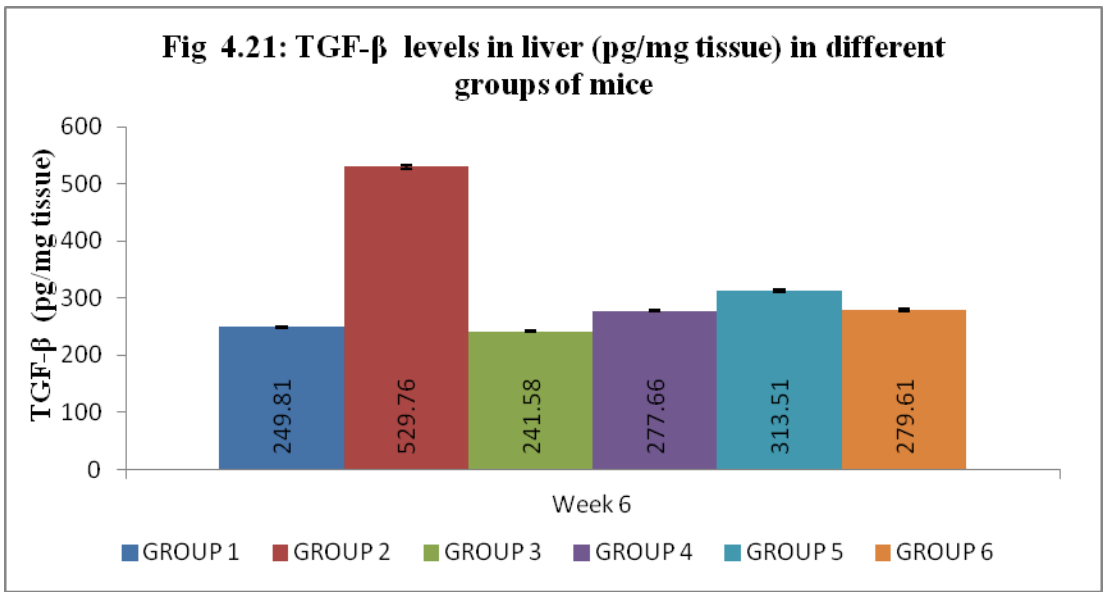


Fig 4.21: TGF-β levels in liver (pg/mg tissue) in different groups of mice



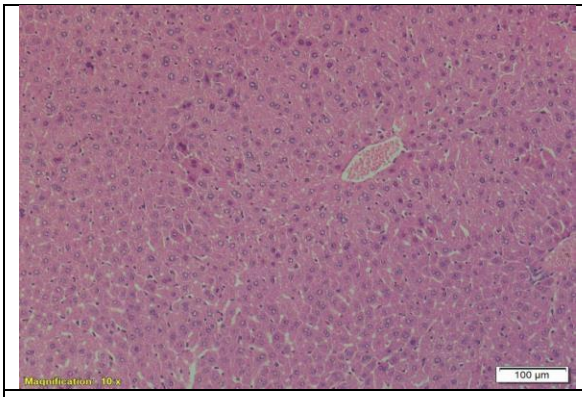


Fig.4.22: Photomicrograph of liver showing normal architecture with normal arrangement of hepatic cords and uniform size nucleus in the hepatocytes in group-1 (H & E 10 x).

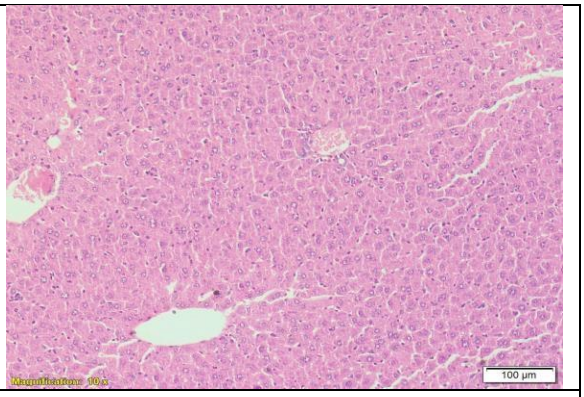


Fig.4.23: Photomicrograph of liver showing Hepatocytes showing uniform hepatic cord with uniform size hepatocytes with normal appearance of Kupffer cells in group-3 (H & E 10 x).

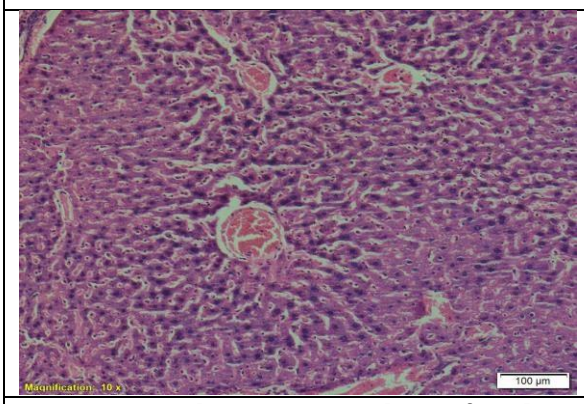


Fig.4.24: Photomicrograph of liver showing moderate to severe congestion of portal vein (PV) and central vein (CV), narrrowing of hepatic cords, dilated sinusoids and pycnotic nuclei of hepatocytes in group-2 (H & E 10 x).

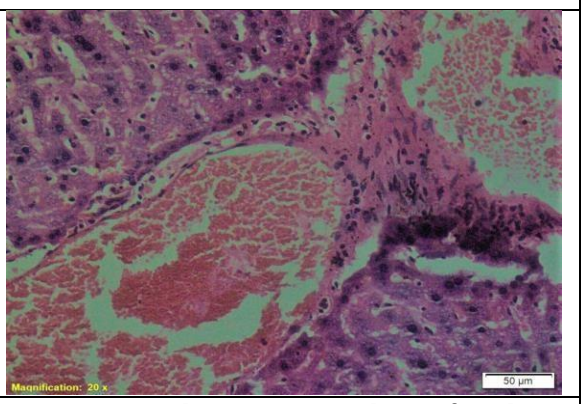
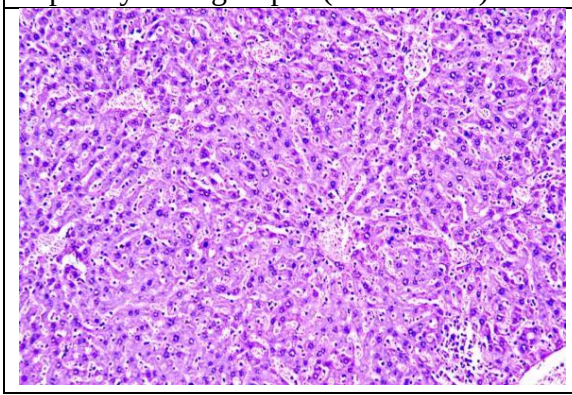
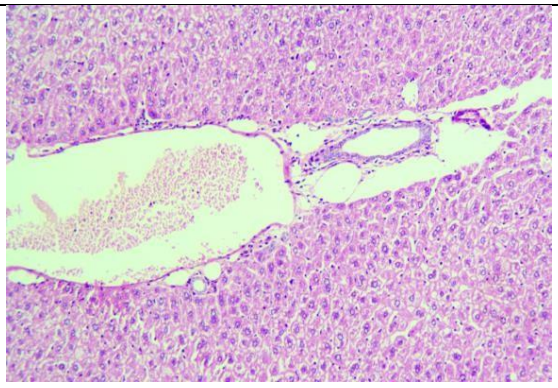


Fig.4.25: Photomicrograph of liver showing mild focal fibrosis of periportal area with mild vasculitis and mild vacoular degeneration of hepatocytes in group-2 (H & E 20 x).





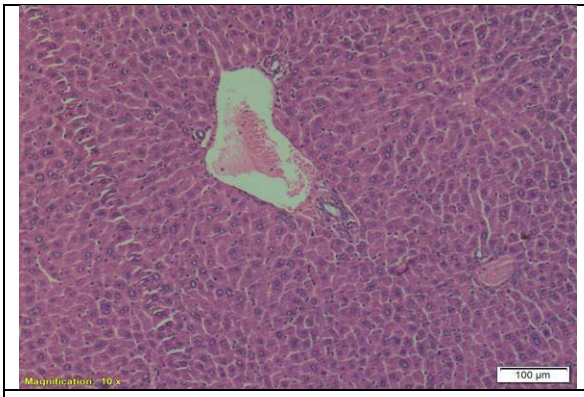


Fig.4.28: Photomicrograph of liver showing few binucleated hepatocytes and majority of cells showing normal appearance of hepatocytes with normal nuclei and portal triad in group-4 (H & E 10 x).

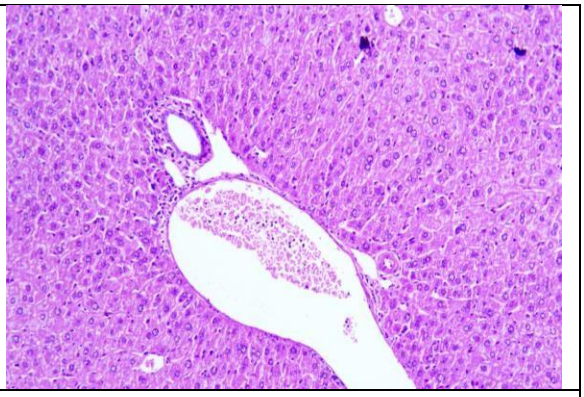


Fig.4.29: Photomicrograph of liver showing mild fibrous tissue proliferation around the portal triad and moderate congestion of portal vein in group-4 (H & E 100 x).

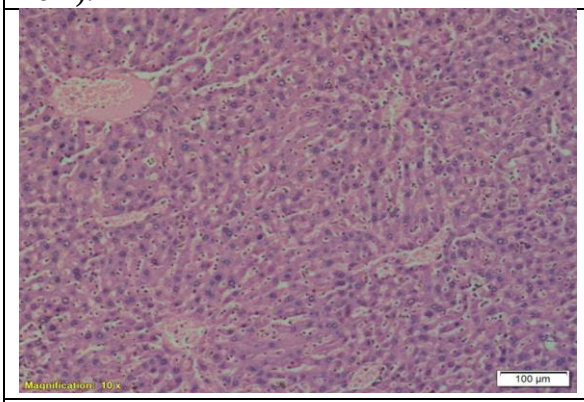


Fig.4.30: Photomicrograph of liver showing normal hepatic cords, with mild congestion of central vein (CV) and sinusoids and mild proliferation of Kupffer cells in group-5 (H & E 10 x).

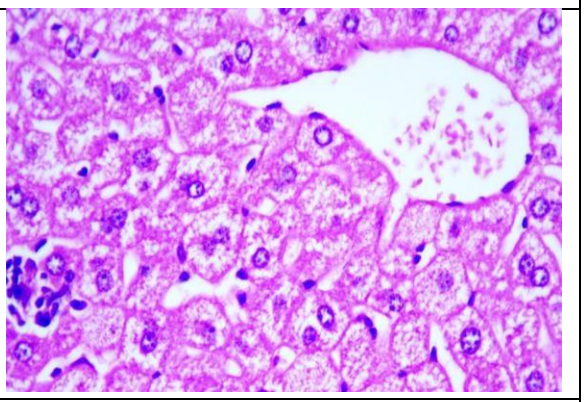


Fig.4.31: Photomicrograph of liver showing severe cellular swelling, mild congestion of central vein and focal infilteration of mononuclear cells in group-5. (H & E 400 x).

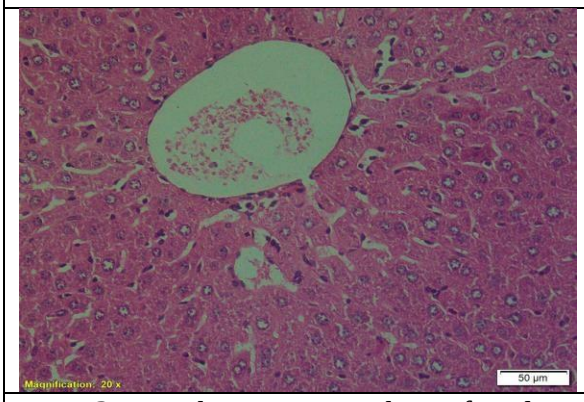


Fig.4.32: Photomicrograph of liver showing mild proliferation of Kupffer cells , mild dilatation of sinusoidal and uniform size hepatocytes in group-6 (H & E 20 x).

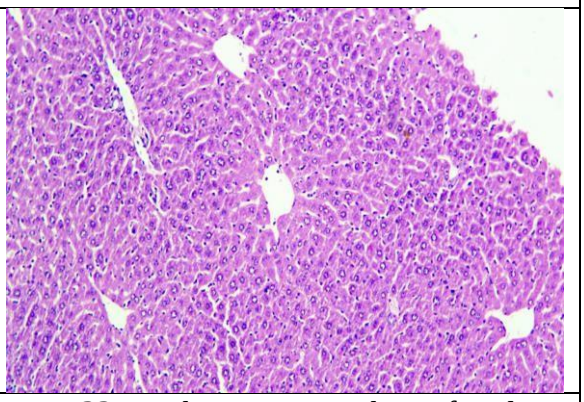


Fig.4.33: Photomicrograph of liver showing near to normal architecture with mild sinusoidal dilatation in group-6 (H & E 100 x).

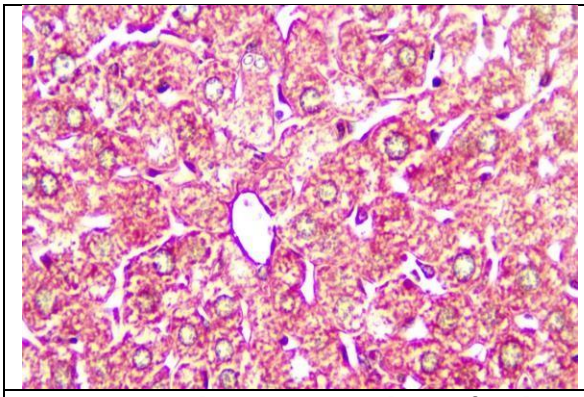


Fig.4.34: Photomicrograph of liver showing normal architecture with specific staining of basement membrane of central vein in group-1 (MTS 400 x)

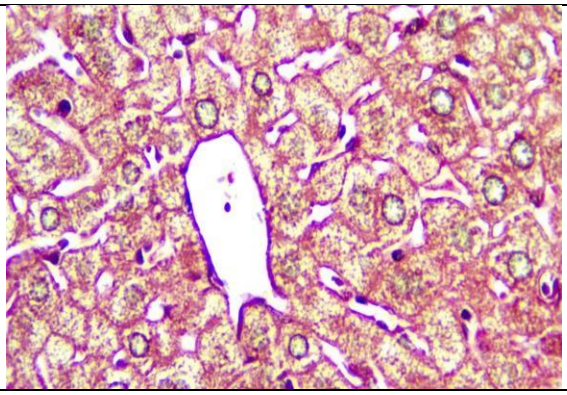


Fig.4.35: Photomicrograph of liver showing normal architecture with specific staining of basement membrane of central vein in group-3 (MTS 400 x)

Fig.4.36: Photomicrograph of liver showing mild fibrosis in periportal area is seen in group-2 (MTS 20 x)

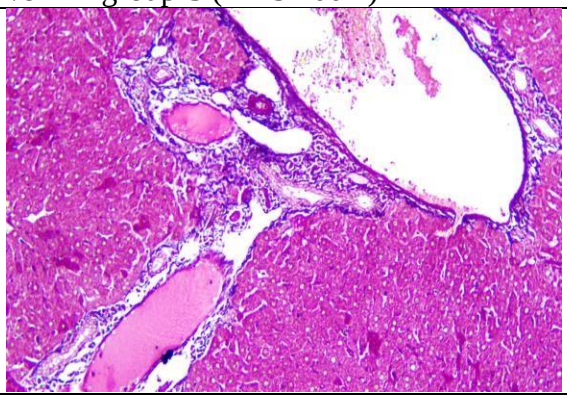


Fig.4.37: Photomicrograph of liver showing moderate proliferation of fibrous tissue around the portal traid along with moderate bile duct proliferataion and mild congestion of portal vein in group-2 (MTS 100 x)

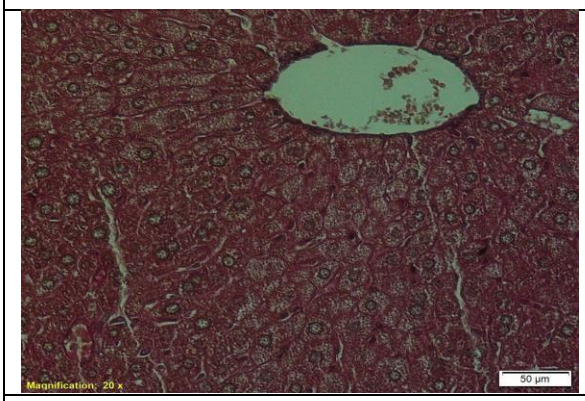


Fig.4.38: Photomicrograph of liver showing normal architectural details of liver with central vein in group-4 (MTS 20 x)

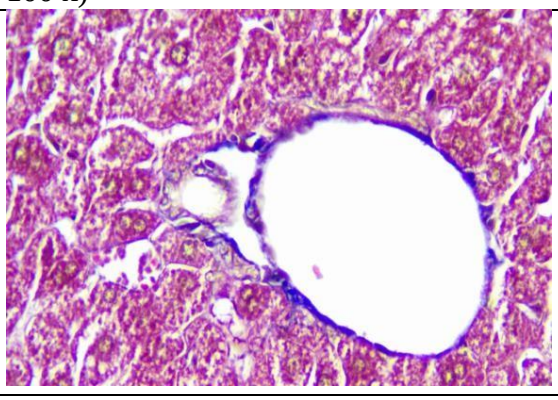


Fig.4.39: Photomicrograph of liver showing very mild proliferation of fibrous connective tissue around the portal vein in group-4 (MTS 400 x)

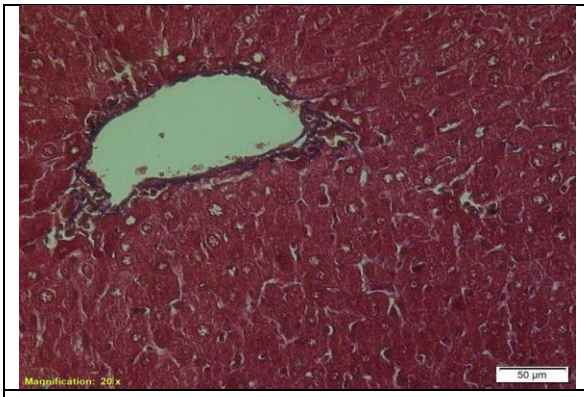


Fig. 4.40: Photomicrograph of liver showing hepatic cords and sinusoids in group-5 (MTS 20 x)

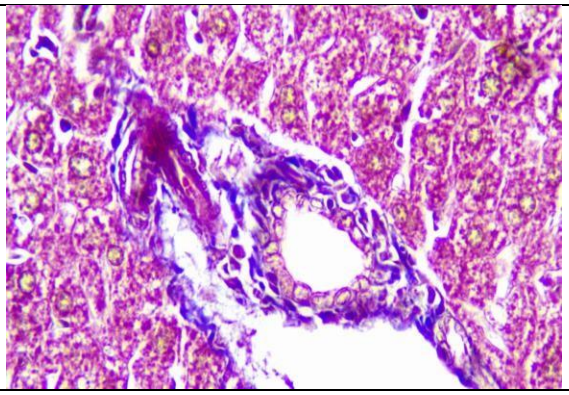


Fig.4.41: Photomicrograph of liver showing moderate proliferation of fibrous connective tissue around the bile duct in group-5 (MTS 400 x)

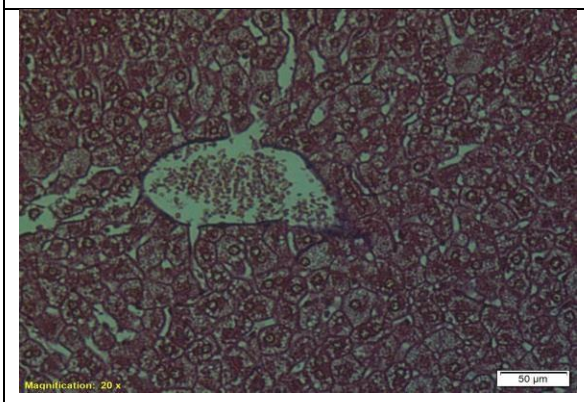


Fig.4.42: Photomicrograph of liver showing normal architecture of liver in group-6 (MTS 20 x)

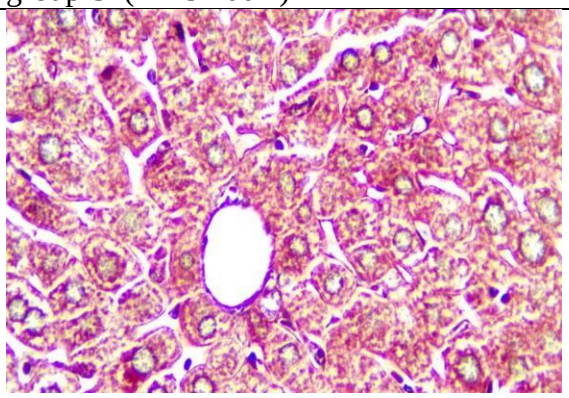


Fig.4.43: Photomicrograph of liver showing normal architecture with specific staining of basement membrane of central vein in group-6 (MTS 400 x)

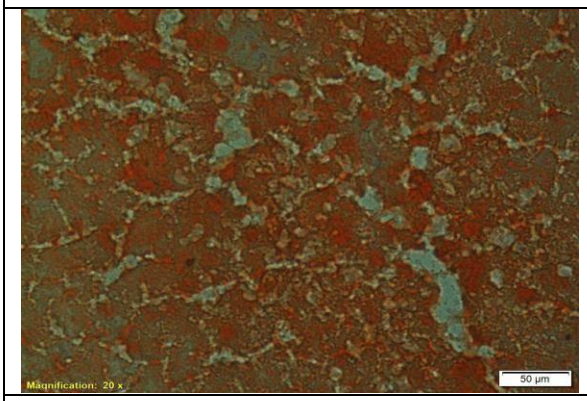


Fig.4.44: Photomicrograph of liver showing mild fatty changes and mild dilated sinusoids in group-2 (Oil Red O 20x)

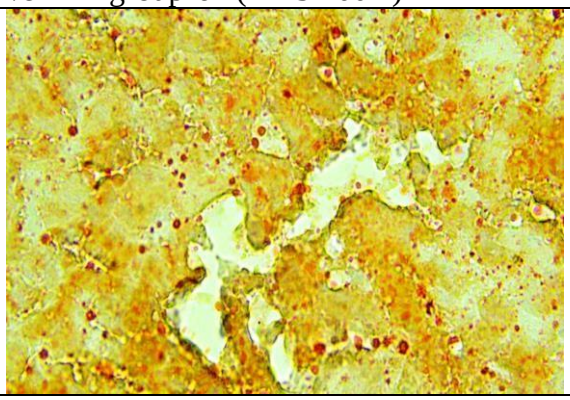


Fig.4.45: Photomicrograph of liver showing moderate micro and macro vesicular fatty change in group-2 (Oil Red O 100x)

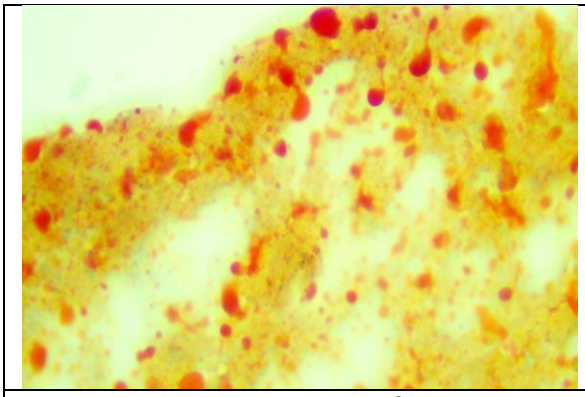


Fig.4.46: Photomicrograph of liver showing moderate micro and macro vesicular fatty change in group-2 (Oil Red O 400x)

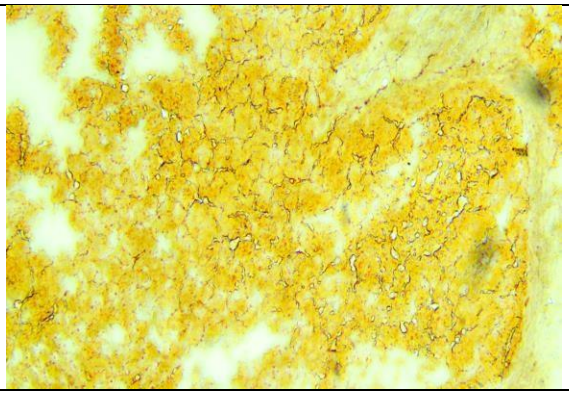


Fig.4.47: Photomicrograph of liver showing negative for fat stain in group-3 (Oil Red O 100x)

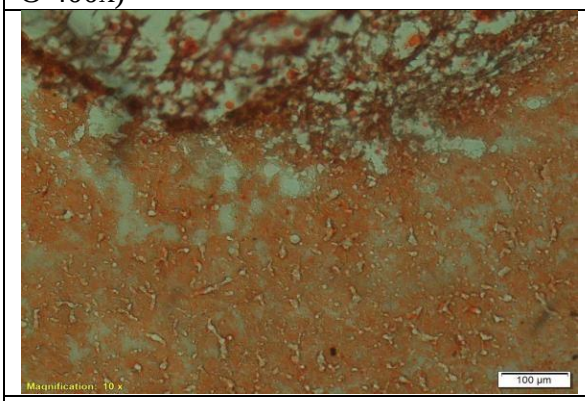


Fig.4.48: Photomicrograph of liver showing Negative for fat in group-4 (Oil Red O 20x)

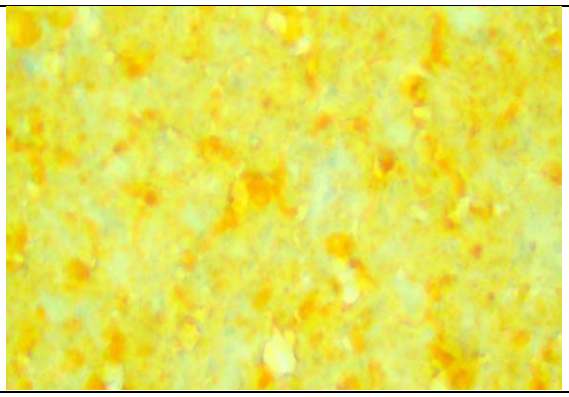


Fig.4.49: Photomicrograph of liver showing very mild positive for fat in group-4 (Oil Red O 400x)

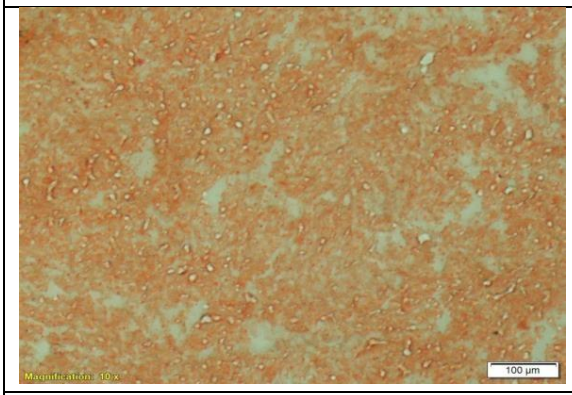


Fig. 4.50:Photomicrograph of liver showing mild fatty change in group-5 (Oil Red O 20x)

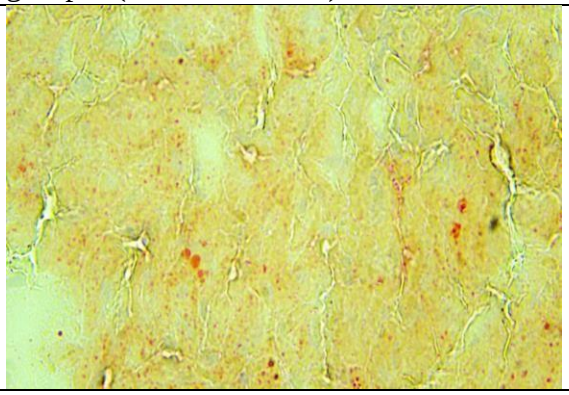


Fig. 4.51: Photomicrograph of liver showing moderate positive for fat in group-5 (Oil Red O 100x)

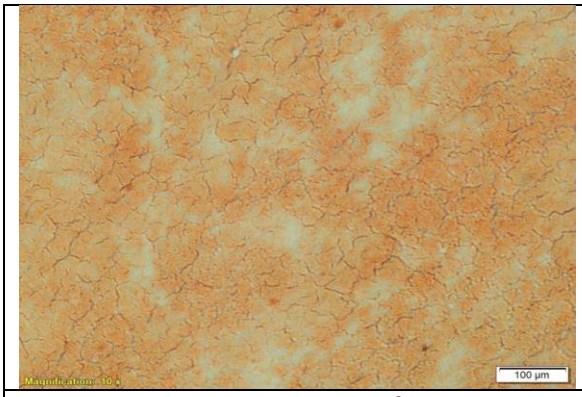


Fig. 4.52:Photomicrograph of liver showing no fat in group-6 (Oil Red O 20x)

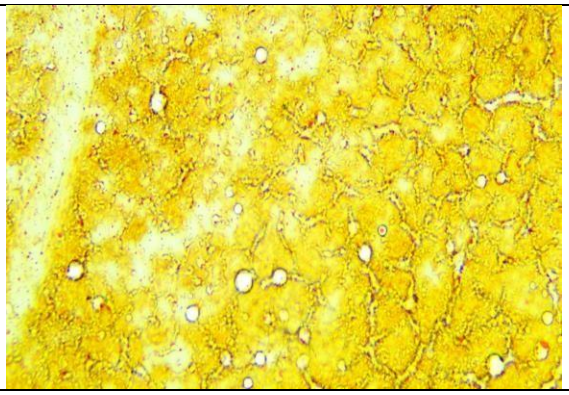


Fig. 4.53: Photomicrograph of liver showing mild positive for fat in group-6 (Oil Red O 100x)

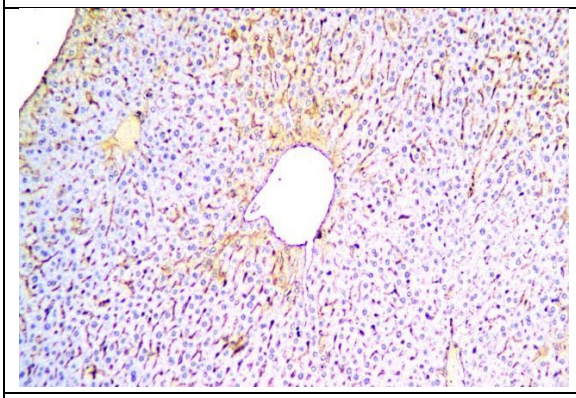


Fig.4.54: Photomicrograph of liver not showing immunoreactivity towards Bcl-2 in group-1 (IHC 100 x).

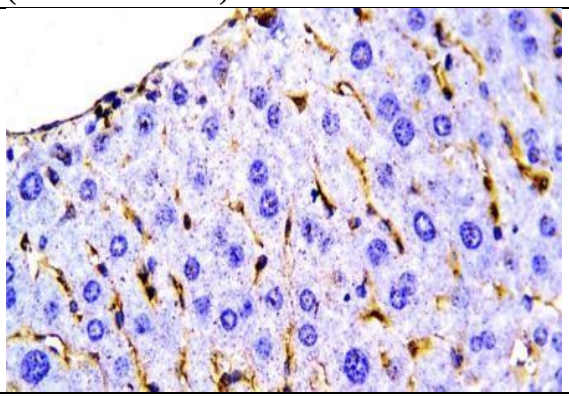


Fig.4.55: Photomicrograph of liver not showing immunoreactivity towards Bcl-2 in group-1 (IHC 400 x).

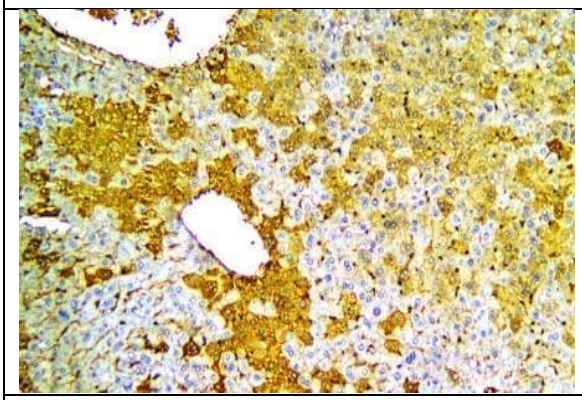


Fig.4.56: Photomicrograph of liver showing intense cytoplasmic reactivity for Bcl-2 in group-2 (IHC 100x).

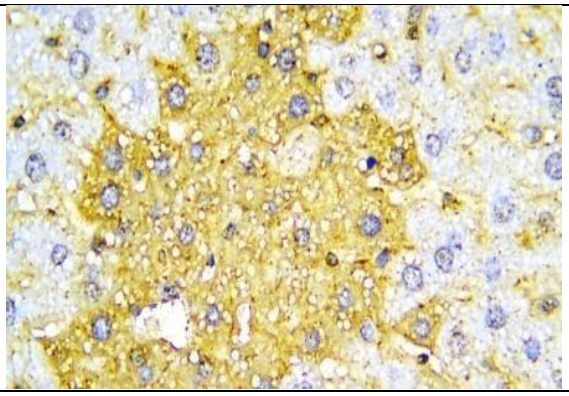


Fig.4.57: Photomicrograph of liver showing intense cytoplasmic reactivity for Bcl-2 in group-2 (IHC 400 x).

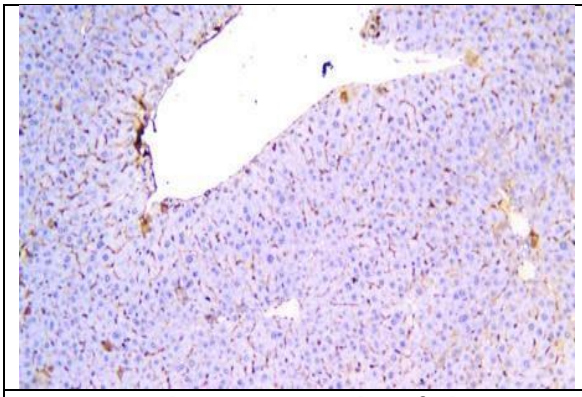


Fig.4.58: Photomicrograph of liver not showing immunoreactivity towards Bcl-2 in group-3 (IHC 100 x).

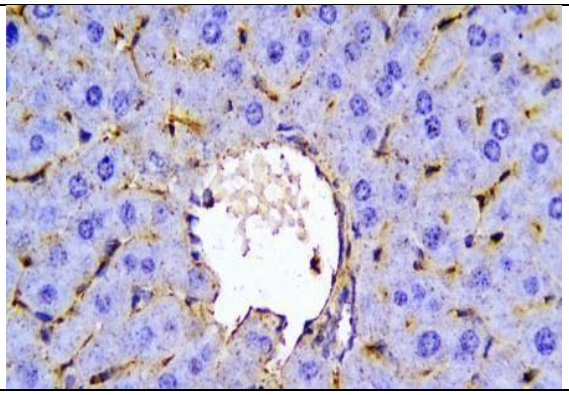


Fig.4.59: Photomicrograph of liver not showing immunoreactivity towards Bcl-2 in group-3 (IHC 400 x).

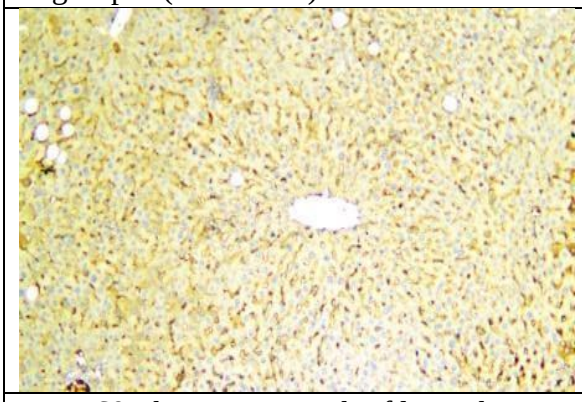


Fig.4.60:Photomicrograph of liver showing very mild cytoplasmic immunoreactivity for Bcl-2 in group-4 (IHC 100 x).

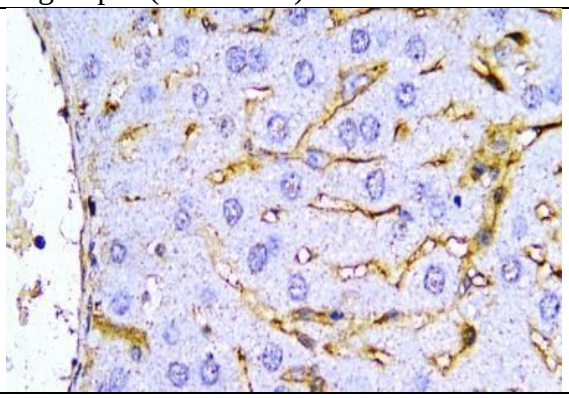


Fig.4.61 Photomicrograph of liver showing very mild cytoplasmic immunoreactivity for Bcl-2 in group-4 (IHC 400 x).

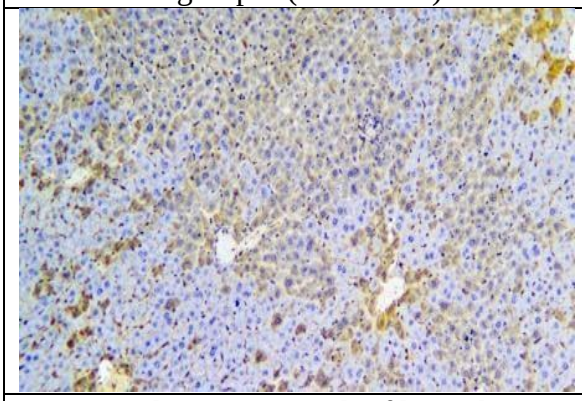


Fig.4.62:Photomicrograph of liver showing moderate cytoplasmic immunoreactivity for Bcl-2 in group-5 (IHC 100 x).

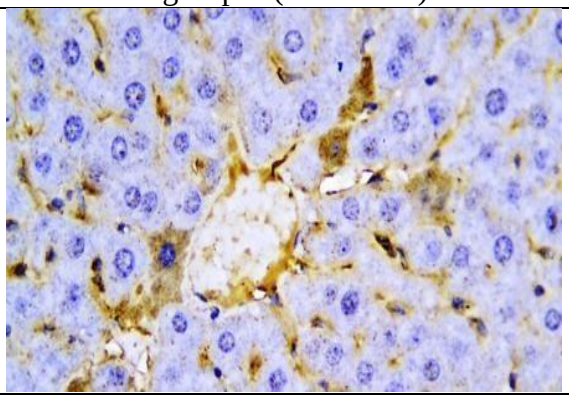


Fig.4.63:Photomicrograph of liver showing moderate cytoplasmic immunoreactivity for Bcl-2 in group-5 (IHC 400 x).

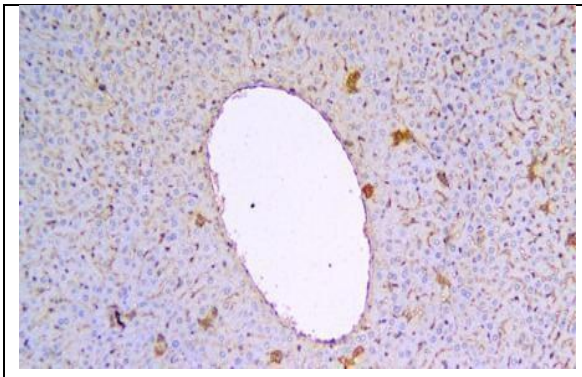


Fig.4.64:Photomicrograph of liver showing very mild cytoplasmic immunoreactivity for Bcl-2 in group-6 (IHC 100 x).

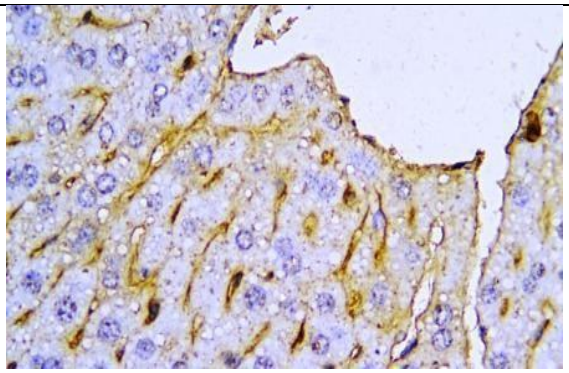


Fig.4.65:Photomicrograph of liver showing very mild cytoplasmic immunoreactivity for Bcl-2 in group-6 (IHC 400 x).

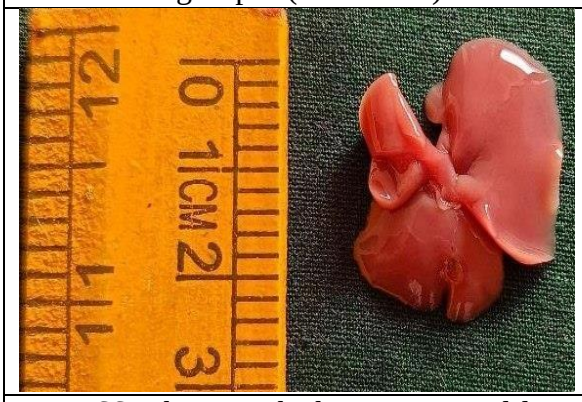


Fig.4.66: Photograph showing normal liver in Group 1

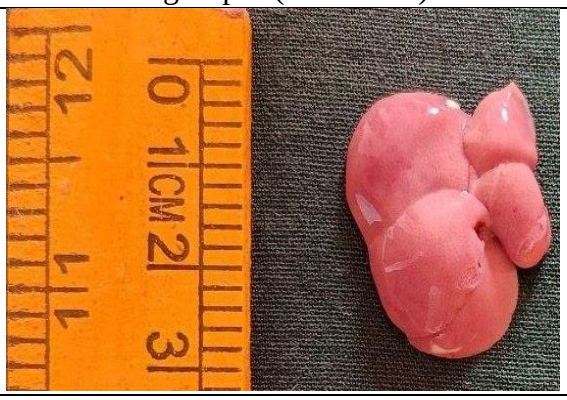


Fig.4.67: Photograph showing fatty liver in Group 2

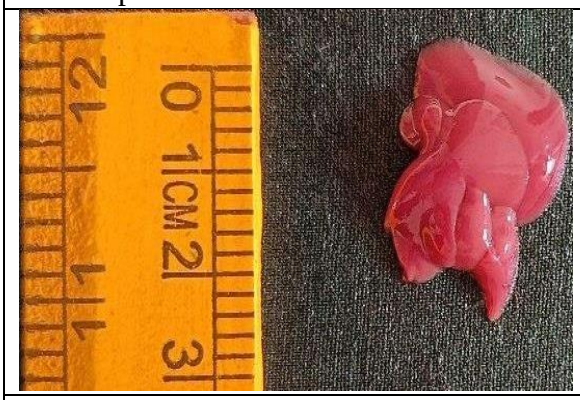


Fig.4.68: Photograph showing normal liver in Group 3

Fig.4.69: Photograph showing near to normal with edema in liver of Group 4

Fig.4.70: Photograph showing mild congestion and edema in liver of Group 5

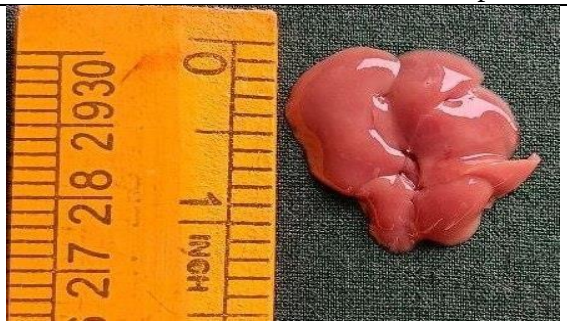


Fig.4.71: Photograph showing normal liver in Group 6

CHAPTER V

DISCUSSION

Nonalcoholic fatty liver disease (NAFLD) is metabolic syndrome that is associated with steatosis and progresses into nonalcoholic steatohepatitis (NASH), fibrosis and cirrhosis (Ishtiaq *et al.*, 2019). Liver disorders are considered to be, major health concern, as it is the important vital organ in clearing the metabolites from the body. NAFLD is considered as a common risk factor associated with metabolic disorders such as obesity, insulin resistance (IR), and T2DM. Despite many advance medical insights, the prevalence of NAFLD is steadily progressing parallel to obesity and global prevalence was estimated to be 25.24 % a hallmark of chronic liver disease (Tsuchida *et al.,* 2018). NAFLD is of clinical interest because of its association with end-stage liver disease *i,e* cirrosis and hepatocellular carcinoma leading to mortality. Many a times as liver is silent organ, long-term chronic liver condition goes unnoticed for years. Day and James (1998): Steatohepatitis: A Tale of Two ‘‘Hits’’? Opined two important hits in progression of NASH first hit (accumulation of fat: fatty liver) and second hit (hepatocellular damage attributed to oxidative stress and abnormal cytokine secretion). Research efforts in NAFLD have been aimed towards disease progression and pathophysiology facilitating drug discovery. Various animal models have been developed involving diet. Diet modulations leading to NAFLD and chemical-induced models have an advantage of convenience and establishment of both mouse and rat models with evidence of fatty liver /fibrosis. In the present study, NAFLD was induced in C57BL/6 mice with high fat diet and CCl_4 for a period of 6 weeks

Pharmacological agents have been studied as alternative treatment options in patients suffering with NAFLD/NASH as they often suffer with obesity, insulin resistance, hyperglycaemia, dyslipaedemias and hypertension. Potential targets treatments include, antioxidants, lipid lowering and anti-inflammatory therapies as gold standards for treatment of NAFLD/NASH. Therefore, it's important to focus on nutritional intake of dietary supplements to prevent and understand the mechanisms involved in the progression of steatosis to hepatosteatosis.

Lactoferrin an iron binding glycoprotein from transferrin family is a major component of milk and is present in most biological fluids. Its wide distribution determines its pleiotropic property as antioxidant, antiinflamatory, antimicrobial and immunomodulatory effect (Sun *et al.*, 2016). In the present study BLF is studied as neutraceutical at two dose levels 100mg/Kgb.wt and 300mg/Kgb.wt to understand the mechanisms and its efficacy in HFD and CCl_4 induced NAFLD.

Previous studies conducted indicated that disorders in hepatic microcirculation may play a important role in progression of NAFLD and the degree of NASH is inversely proportional to hepatic microcirculatory blood flow **(**Gracia-Sancho *et al.*, 2013 and Pereira *et al.*, 2021). Decrease in blood flow contributes to tissue damage by ROS generation and lipid per oxidation (Rosenstengel *et al*., 2011 and Schleicher *et al*., 2014). Statins being the most efficient conventional agents for reducing plasma cholesterol are also appreciated for their good tolerance. The most severe adverse effect of statin therapy is myotoxicity. Its various forms include myopathy, myalgia, myositis and rhabdomyolysis (Joy and Hegele, 2009**).** Current clinical guidelines support the use of statins for treatment of dyslipaedemias and myocardial infarction in patients with

NAFLD. In the present study Simvastatin is used @ 10mg/Kg b.wt . Statins are reported to enhance the microcirculatory function and have hypolipaedemic effect (Schierwagen *et al.,* 2017). In view of the shown beneficial effects, the present study was designed and carried out to evaluate the efficacy of BLF and Simvastatin in HFD+CCl_4 induced NAFLD in C57BL/6 mice for a period of six weeks.

The body weights are the preliminary marker to assess the eventuality of the HFD- induced hyperlipidemia. In the present study, the positive control (group 2; HFD+CCl_4) animals had shown significant increase in the average body weight (g) when compared to the other groups at respective time intervals in the course of 6 weeks. The results of the present study are consistent with the report of **(**Chheda *et al.,* 2014**).** Animals with standard diet (group 1) showed normal range of body weights throughout the study. The simvastatin-treated animals (group 6) showed a decrease in body weight when compared with group 2. The BLF treatment groups (3, 4 and 5) revealed significant decrease in body weight but lesser in comparison to group 2 as BLF have been reported to reduce hyperlipidemia and inflammation (Xiong *et al.,* 2018). BLF has been reported to reduce secretion of leptin and corticosterone (McManus *et al*., 2015).

The absolute weights and relative weights of liver in group 2 mice were significantly increased when compared to the remaining groups on 6^{th} week of study. The results are similar to the findings of Gaemers *et al*. (2011) who reported increase in weights of liver and relative liver weights. The increase in liver weights might be attributed to fatty liver and accumulation of lipids and inflammatory cells. The simvastatin-treated animals (group 6) showed a decrease in weights of liver and relative liver weights when compared with group 2 were similar with the findings of Souza *et al*.

(2020). The BLF treatment groups (3, 4 and 5) revealed significant decrease in weights of liver and relative liver weights but lesser in comparison to group 2 as BLF had decreased fat accumulation, oedema and infiltration of inflammatory cells in liver (Xiong *et al.,* 2018).

The serum glucose level was high in group 2, which may be attributed to the HFD-induced obesity throughout the experimental period. These findings correlate with the report of Jaisheela *et al.,* (2021) and Ishtiaq *et al*., (2022). Group 6 mice treated with simvastatin showed decrease in glucose level when compared with Group 2 the results are in collaboration with Pereira *et al*. (2022).

The present study demonstrated that HFD+CCl_4 (group 2) caused a significant reduction in HDL and a significant elevation in groups 3, 4 and 5 when compared with those in control group 1 and simvastatin-treatment group 6. The present results indicated that the reduction in serum lipid profile and elevation of HDL in group 3, 4 and 5 treated with BLF may be because of the enhancing activity of enzymes involved in bile acid synthesis and its excretion. This effect was associated with an improvement in lipid and lipoprotein profiles. The tendency to the normalization of lipid and lipoprotein levels in mice could be attributed to reducing the synthesis and absorption of cholesterol and increasing cholesterol excretion (Chen *et al.,* 2019). It was also noted that simvastatin-treated animals (group 6) have shown, the serum lipid profile almost similar to that of control animals. HMG-CoA reductase catalyzes the reduction of HMG-CoA to CoA and mevalonate, the rate-limiting reaction in the de novo synthesis of cholesterol. It plays an important role in reducing cholesterol levels in the blood and peripheral tissues there by inhibits atherosclerotic plaque formation in the aorta (Young *et al.,* 2004). The findings

of the present study showed decreased levels of cholesterol and triglycerides in the treatment groups 4, 5 & 6. Statins reduce cholesterol levels as inhibitors of HMGCR activity. BLF may reduce cholesterol absorption in the small intestine and increase fecal excretion. The results of the present study in Oil in red O staining showed moderate fat droplets in group 2 and in the treatment groups showed near to normal architecture, our results are in consistent with Nakamura *et al*. (2017).

Insulin resistance breaks down the triglycerides by specific enzymes like, ATGL (adipose TG lipase) HSL (hormone sensitive lipase) and MGL (monoglyceride lipase), which induce flux of FFA to liver. HFD absorbed in the gut are incorporated as TG into chylomicrons and promote gene expression of transcriptional factors Sterol Regulatory Element Binding Protein 1c (SREBP1c) and Carbohydrate Response Element Binding Protein (ChREBP) activated *via* insulin signaling responsible for de nova lipogenesis (Pierantonelli and Svegliati-Baroni, 2019) are associated with insulin resistance, dyslipaedemias and metabolic/cardiovascular diseases.

Excess fatty acids trigger ROS. FFA is oxidized to carbon monoxide and water to produce energy and reduce lipid content in liver. When the striking balance is lost between the production of ROS and antioxidant defense mechanisms there will be oxidative stress. In progression of NASH, FFA overload up regulates mitochondrial and peroxisomal beta oxidation and increased ROS and also endogenous antioxidant system is less efficient in steatotic liver. ROS also induce lipid per oxidation which further leads to HSC. Increased FFA beta oxidation can uncouple hepatic TCA cycle function causing mitochondrial dysfunction (Neuschwander, 2010 and Yesilova *et al*., 2005**).**

Mitochondrial dysfunction due to a lipid overload may induce an impairment of electron transport chain, resulting in an “electron leakage.” The reaction between oxygen and protons catalyzed by cytochrome C oxidase (VI complex) is impaired, and electrons may interact directly with oxygen forming ROS. Furthermore, the generation of mitochondrial membrane potential is reduced following the reduction of proton extrusion from the matrix, weakening the activity of ATP synthase (Basaranoglu *et al.,* 2013**).**

In addition to prooxidant mechanism, in an experimental model of NAFLD/ NASH, a decreased activity of several detoxifying enzymes was observed. Glutathione peroxidase (GPx) activity is reduced probably in consequence of GSH depletion and impaired transport of cytosolic GSH into the mitochondrial matrix .The results of the present study GSH activity is increased in treatment groups (4,5 & 6) the result of the present study are in agreement with Chen *et al*. (2016) .

In our study, the antioxidant defenses such as SOD, CAT and GSH of liver were showing significant decrease in HFD+CCl_4 (group 2) when compared to other groups. BLF treated groups (3, 4 and 5) also had shown a significant elevation when compared to group 2. Oxidative stress and antioxidants play a major role in the pathogenesis of NAFLD (Matsunami *et al.,* 2010). Increase in fatty acids induces oxidative metabolism, causing oxidative stress and inflammation leading to ROS formation damaging hepatocytes and producing oxidative damage products, of lipid peroxidation the MDA, as those reported earlier in both human and experimental NAFLD (Zou *et al*., 2006). In the present study the MDA levels in treatment groups 4, 5 and 6 treated with BLF and simvastatin decreased significantly compared to Group 2 the results of the study are in agreement with Farid *et al*. (2021). Further the precise mechanism of attenuation of

oxidative stress by BLF is not clear but speculated probably through its regulation of redox cycling and hydroperoxide decomposition (Chen *et al*., 2016). Oxidative stress can also not only lead to cell membrane injury but also the destruction of NO. Thus, the natural antioxidant properties of NO are lost and oxidative stress continues unabated.

SOD and CAT are endogenous antioxidant defenses that protect the animal from ROS-mediated oxidative damage as NAFLD progress, antioxidant reserves may be depleted. In the present study increased antioxidant enzyme activity may be an indicative of adaptive response that counteracts lipid peroxidation and ROS generation in NAFLD. Results of the present study are in agreement with Pereira *et al*. (2022) and Farid *et al*. (2021). The oxidative stress is opined as key partner in pathophysiology of NAFLD. Moreover, these theories found a "clinical" confirmation from the studies that evidenced the association between metabolic syndrome and also its components (hypertension, obesity, and dyslipidemia) taken individually. Moreover, the activity of glutathione peroxidase, MnSOD, and catalase seem to be low in NASH, so that the capability of the mitochondria to reduce ROS levels is reduced (Delli Bovi *et al*., 2021)

In the development of NASH, oxidative Stress probably occurs not only due to the saturation of the antioxidant machinery secondary to the increased pro-oxidant species production and its direct insult. Overall, these events appear involved in the diffusion of ROS and reactive nitrogen species (RNS) into the extracellular space, perpetuating intracellular and tissue damage. (Delli Bovi *et al*., 2021).

In the present study, the levels of the pro-inflammatory cytokines such as TGF-β and TNF - α had shown a significant elevation in the positive control group 2 when compared to the other groups, which could be explained in terms of the inflammatory changes and oxidative stress, as both TNF-α and IL-10 are pleiotropic cytokines that play a very important role in the initiation and maintenance of inflammation and immune responses (Rutgeerts *et al.,* 2005). Inflammatory cytokines TGF-β stimulates its own production from myofibroblasts, differentiation and activation (Xu *et al.*, 2012). From the results of present study the BLF treatment groups, group 4 (dosed @ 300mg/Kg b.wt) showed better attenuation by lowering TGF-β compared to group 5 (BLF dosed @ 100mg/ Kg b.wt) & 6 (simvastatin @10mg/ Kg b.wt). The results of cytokines are also in correlation with histopathology results of the present study. Group 2 showed moderate to severe congestion of portal and central vein narrowing of hepatic cords, dilated sinusoids and pycnotic nuclei in hepatocytes. Severe diffuse vacuolation with moth eaten appearance of cytoplasm and nuclear condensation. Further, BLF treatment group 5 (100mg/ Kg b.wt) showed normal hepatic chords with congestion of central vein and sinusoids mild proliferation of Kupffer cells and focal infiltration of mononuclear cells. Group 4 (300mg/ Kg b.wt) showed few binucleated hepatocytes and majority of cells showing normal appearance of hepatocytes with normal nuclei and portal triad and regeneration of hepatocytes. Group 6 showed few binucleated hepatocytes and majority of cells showing normal appearance of hepatocytes with normal nuclei and portal triad and regeneration of hepatocytes with many mitotic figures and mild fatty change. The results of our study are in agreement with (Naiki-Ito *et al.*, 2020).

Animals treated with BLF (groups 3, 4 and 5) showed a decrease in the pattern of the TGF- β and TNF- α, which can be attributed to the antioxidant properties exhibited by BLF in the system. The results of the present study are consistent with Chen *et al*. (2016). Pro-inflammatory markers TNF- α, TGF- β and nuclear factor kappa B increase inducible nitric oxide synthase (iNOS) and consequently NO production. More NO production in HFD animals supports more intense inflammation state in liver; the findings of the present study are consistent with Santi *et al.* (2016). NO plays an important role in pathophysiology of NAFLD. In liver NO generated by eNOS will responsible for liver homeostasis and protect against any pathology. iNOS is induced by immune stimuli and inflammatory stimuli. TNF-α may induce iNOS expression (Bryan *et al*., 2009). Our findings are in correlation with the above finding by elevation in TNF- α and NF-*κ*B in positive control group 2 and reduced in treatment groups 4, 5 & 6 respectively, together with an increase in the NO metabolite nitrite in group 2 and decrease in simvastatin and BLF treatment groups. Souza *et al*. (2020) reported that simvastatin is a known anti-inflammatory drug mainly through the inactivation of NF-*κ*B, a key activator of cytokine transcription and through the resulting decrease in the expression levels of reactive oxygen species, inhibition of matrix metalloproteinases and decrease of proinflammatory cytokines including TNF- α.

The anti-inflammatory cytokines such as IL-10 had shown a significant decrease in the positive control group 2 when compared to the other groups (Elshopakey *et al.*, 2021). Animals treated with BLF (groups 3, 4 and 5) showed an increasing pattern of the IL-10 levels (Farid *et al*., 2021). These observations corroborate with reported upregulation of anti-inflammatory markers that inhibit synthesis and release of pro-

inflammatory markers (Cassatella *et al.,* 1993). Liu *et al.* (2006) reported that the deficiency of the cytokine IL-10 accelerates atherosclerosis, whereas it's over expression inhibits advanced lesions, decreases cholesterol and phospholipid oxidation products in the aorta along with monocytic activation.

The ALT and AST activity is reported to be sensitive marker for hepatotoxicity due to its cytoplasmic location. Any damage to hepatocyte leads to release of this enzyme into circulation. Statins have been reported to inhibit the synthesis of mevalonate, a precursor of ubiquinone, which is a central component of mitochrondrial respiratory chain (De Pinieux *et al.,* 1996) in liver. Mitochondrial dysfunction could also be explained by membrane damage that was induced following inhibition of HMG Co A reductase. In the findings of the present study simvastatin group 6 showed elevated activity of serum transaminases compared to treatment groups (4 and 5) this could be attributed to the hepatic injury by statin and inhibition of metabolizing enzymes. Correspondingly, histopathology liver revealed mild increase in number of Kupffer cells and mild dilatation of sinusoids. BLF treated group (5) showed few binucleated hepatocytes and majority of cells showing normal appearance of hepatocytes with normal nuclei. BLF group 4 showed normal hepatic chords with mild congestion of central vein and sinusoids, mild proliferation of Kupffer cells.

The normal levels of total bilirubin (TBR) and GGT activities were increased in the group 2 (Chang *et al.,* 2021 and Jaisheela *et al.,* 2021) due to cellular damage and loss of functional integrity of cell membrane in liver (Drotman and Lawhorn, 1978). It was observed that, concurrent treatment with BLF in group 4, 5 and simvastatin in group 6 decreased the TBR and GGT values (Asghar *et al*., 2019). BLF with its anti-oxidant

properties might have protected the hepatic tissue from NAFLD induced injury. Also there was decrease in production of proteins due to damage of hepatocytes, and hence total protein levels are reduced in group 2 (Jaisheela *et al.,* 2021 and Elshopakey *et al.,* 2021) as liver is the major source for production of proteins. These results may be due to the impaired liver function and hepatic cell damage caused by CCl_4 (Kazeem *et al*., 2011).BLF treated groups 3,4 and 5 showed significant elevation in protein (Aoyama *et al*., 2022) unlike NAFLD intoxicated group due to its antioxidant and hepatoprotective efficacy.

NAFLD/NASH is represented as a source of excessive lipid accumulation into the liver, described as increased visceral adipose tissue lipolysis, de nova lipogenesis activation and HFD (Donnelly *et al.,* 2005)**.** NASH can be from many pathological stimuli from other organs like molecules secreted from adipose tissue and gut which promote inflammation and fibrinogenesis *via* activation of resident Kupffer cells. It is difficult to induce fibrosis with short duration of experimental studies, the results of the present study for 6 weeks showed only mild fibrosis in group 2 as evident from histopathology discussed, the Masson's trichome staining in group 2 revealed moderate proliferation of fibrous tissue around portal triad. The Oil Red O special stain also revealed moderate micro and macro vesicular changes, where as treatments showed only mild positive for fat.

In a lipotoxicity, the toxic lipids accumulated cause ER stress through inositol-requiring enzyme-1 (IRE1) and protein kinase-like ER eukaryotic initiation factor-2α kinase (PERK), pathway it is also linked to chronic inflammation through excessive production of reactive oxygen species (ROS) and the activation of NF-κB (Win *et al.,*

2015). ROS production can also promote hepatic inflammation by increasing the secretion of TNF α from hepatocytes and Kupffer cells thus, up regulating the synthesis of inflammatory cytokines (Kern *et al.,* 1995) as evident from the findings of present studies and HP of liver from positive control and treatment groups. IRE1 can bind to a protein TNF- α related apoptosis-inducing ligand (TRAIL) which is functioning as a ligand and promote apoptosis (Wiley *et al.,* 1995). In the presence of nutrient excess, macrophage-associated hepatic and adipose tissue inflammation uses TNF- α related apoptosis-inducing ligand receptor signaling to induce liver injury and fibrosis (Idrissova *et al.,* 2015).

Furthermore, the ER lumen is the main site of calcium storage, and calcium homeostasis plays a critical role in ER stress (Egnatchik *et al.,* 2014). Saturated fatty acids may induce a disruption of ER calcium store, which can act on mitochondrial membranes blocking ETC through the formation of permeability transition pores for cytochrome C, resulting in an increased ROS production and apoptosis induction (Zhang *et al.,* 2014).

ROS/RNS and lipid peroxidation by products, are responsible for cytokine elevations such as TNF-α and TGF- β, (Delli Bovi *et al.,* 2021). Hepatocyte damage involves a cascade of events leading to NAFLD progression into NASH and cirrhosis: damage associated molecular patterns, discharged from damaged hepatocytes, lead to the release of chemokines and cytokines from Kupffer cells and the recruitment of monocyte derived macrophages. ROS, directly and indirectly, contribute to stellate cell activation

and to a chronic inflammatory response with up-regulation of proinflammatory cytokines (TNF-α, IL-6, and IL-1), apoptosis, and development of hepatic fibrosis (Mann *et al*., 2017).

Intracellular stress lead to apoptosis and release of Cytochrome C from the damaged mitochondrion, which, binds to the APAF-1 (Apoptotic Protease Activating Factor-1) an initiator that triggers a cascade of caspases stimulation resulting in morphological and biochemical changes. Bcl-2(B-cell lymphoma 2) is a cellular protein that inhibits apoptosis. Bcl-2 inhibited apoptosis by preservation of mitochondrial integrity. Bcl-2 is not only localized to mitochondrial membrane but also to the nuclear envelope and the endoplasmic reticulum. The results of present study are further substantiated by immunohistochemistry. The liver sections showed an intense immunopositivity for Bcl-2 in group 2 when compared to other groups. The liver sections of groups 4, 5 and 6 showed mild to very mild immunoreactivity for Bcl-2 when compared to group 2. The results of the present study are in agreement with Li *et al*., (2014) and Kanda *et al*., (2018).

In conclusion, BLF was found to possess the ameliorating action against NAFLD induced by CCl_4 and HFD. The results showed more significant amelioration in group 4 (300 mg/Kg b.wt.), which was evident from histopathology, reducing the pro-inflammatory cytokines and restoration of antioxidant enzymes, possibly *via* inhibiting the activation of NF-κB signaling pathway. These results indicated BLF might act as a preventive agent to prevent hepatic injury, inflammation, and fibrosis in NASH via NF-κB inactivation. However, much research remains to be done to clarify the pathophysiology of NAFLD and to identify selective targets for treatment. The

involvement of cytokines and their receptors in the pathogenesis of NAFLD is only partially understood. The most promising mediators (such as TNF-α and TGF- β) might also require more selective inhibitory drugs to safely improve NAFLD and prevent its progression to NASH and fibrosis, limiting the potential risk of deleterious immune-suppression.

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CHAPTER VI

SUMMARY

An experimental study was conducted to evaluate the therapeutic efficacy of BLF in NAFLD induced by CCl_4 and HFD. Study was performed in C57BL/6 mice as this is the most susceptible animal model towards CCl_4 and HFD induced NAFLD. Forty four healthy male C57BL/6 mice were randomly divided into 6 groups consisting of 6 mice in groups 1 and 3 where as groups 2, 4, 5 and 6 had 8 animals each respectively. For the purpose of statistical analysis (n = 6) has been taken. Group 1 was kept as normal control and fed with standard diet. Remaining groups excepting group 3 were fed with HFD along with CCl_4 @ 0.5 mg/Kg b.wt in olive oil, i.p twice in a week to induce NAFLD. Group 2 was kept as positive control for NAFLD. Group 3 as *per se* BLF @300mg/Kg b.wt p.o and fed with standard diet. Group 4 were treated HFD +CCl_4 @ 0.5mg/Kg b.wt in olive oil i.p twice in a week + BLF @ 300mg/Kg b.wt p.o. Group 5 was given HFD +CCl_4 @ 0.5mg/Kg b.wt in olive oil i.p twice in a week + BLF @ 100mg/Kg b.wt p.o. Group 6 was given HFD +CCl_4 @ 0.5mg/Kg b.wt in olive oil i.p twice in a week + simvastatin @ 10 mg/Kg b.wt p.o. The results of the study are summarized as follows:

The average body weight (g) of animals in all groups with HFD showed significant ($p<0.05$) increase on 6^{th} week when compared to that of group 1 (32.53±2.38) and group 3 (31.51±1.10). Group 2 animals showed significant ($p<0.05$) increase (36.15±6.14) in comparison to groups 4 to 6.

The average liver weight (g) in group 2 (2.12±1.31) was significantly ($p<0.05$) increased on 6th week, when compared to group 1& 3 (1.41±0.41 & 1.33±0.36), while treatment groups 4, 5 and 6 showed significant ($p<0.05$) decrease in liver weight in comparison to group 2. The value of group 4 was comparable to group1.

The relative liver weight (%) in group 2 (5.86±0.29) was significantly ($p<0.05$) increased on 6th week, when compared to group 1& 3 (4.33±0.05 & 4.22±0.08), meanwhile in treatment groups 4, 5 and 6 there was a significant ($p<0.05$) change in relative liver weights when compared to the group 2.

There was a significant ($p<0.05$) increase of serum total cholesterol concentration (mg/dL) in groups 2, 4, 5 and 6 on 4th and 6th week as compared to groups 1 and 3. The cholesterol level of group 2 (182.35±6.26 and 203.66±8.99 on 4th and 6th week, respectively) showed a significant ($p<0.05$) increase when compared to groups 4, 5 and 6. There was a significant ($p<0.05$) increase in the concentration of triglycerides (116.30±13.55, 74.95±9.08, 77.95±7.16 and 74.35±8.79 respectively) (mg/dL) in groups 2, 4, 5 and 6 and significant ($p<0.05$) decrease in HDL (mg/dL) (23.55±0.70,48.96±2.05,46.03±1.24 and 51.11±1.02, respectively) on 4th week when compared to groups 1 and 3. On 6th week, the triglycerides were significantly ($p<0.05$) increased (121.95±11.84) and HDL was significantly ($p<0.05$) decreased (16.80±0.67) in group 2 when compared to all other groups.

Glucose index of group 2 (310.33±5.79) showed a significant ($p<0.05$) increase as compared to group 1 (250.50± 2.47) and group 3 (231.83± 2.15) also significant ($p<0.05$) increase when compared with groups 4 to 6 on 6th week (256.50± 3.14, 259.83±3.74 and 272.33±3.80) respectively.

The activity (IU/L) of ALT (244.18±1.13) and AST (264.93±1.23) in group 2 was significantly ($p<0.05$) higher when compared to all other groups on 6th week. In the treatment groups 4, 5 and 6 there was significant ($p<0.05$) reduction in both ALT and AST when compared to group 2 on 6th week. Group 3 was comparable to group 1 with significant difference.

There was a significant ($p<0.05$) decrease of total protein concentration (g/dL) in groups 2, 4, 5 and 6 on 4th and 6th week as compared to groups 1 and 3. The Protein concentration of group 2 (4.47±0.05 and 4.43±0.05 on 4th and 6th week, respectively) showed a significant ($p<0.05$) decrease when compared to groups 4, 5 and 6. There was a significant ($p<0.05$) decrease in the concentration of total bilirubin (0.13±0.01, 0.17±0.01, 0.19±0.01 and 0.19±0.01 respectively) (mg/dL) in group 3, 4, 5 and 6 and significant ($p<0.05$) decrease in *gamma*-glutamyl transferase (GGT) levels (U/L) (5.15±0.40, 9.46±0.60,10.85±0.50 and 9.87±0.98, respectively) on 4th week when compared to group 2. On 6th week, the total bilirubin and *gamma*-glutamyl transferase were significantly ($p<0.05$) increased (0.38±0.01) and (21.80±0.19) in group 2 when compared to all other groups.

The concentration of GSH (n mol/mg protein) of the liver revealed a significant ($p<0.05$) decrease in group 2 as compared to all other groups. Among the treated groups (4, 5 and 6), group 4 revealed a significant ($p<0.05$) increase (23.15±0.67, 21.24±0.39 and 22.91±0.31 respectively).

The activity of Catalase and SOD (U/mg of protein) of the liver revealed a significant ($p<0.05$) decrease in group 2 when compared to all the other groups at 6th week. Among the treated groups (4, 5 and 6), group 4 revealed a significant ($p<0.05$)

increase (68.91±3.23, 65.78±3.00 and 67.66±3.43 and, 5.87±0.05, 5.12±0.02 and 5.74±0.05 respectively) in Catalase and SOD levels.

The concentration of TBARS (nM MDA/mg protein) of the liver revealed a significant ($p<0.05$) increase in group 2 when compared to all other groups. Among the treated groups (4, 5 and 6), group 4 revealed a significant ($p<0.05$) decrease (2.48±0.04, 2.95±0.06 and 2.67±0.02, respectively) inTBARS.

The nitric oxide levels (µM of nitrite/mg tissue) at the end of experiment revealed a significant ($p<0.05$) rise in group 2 (8.46±0.72) when compared to groups 1 and 3. In the treatment groups 4, 5 and 6 there was a significant reduction when compared with group 2.

The concentration of IL - 10 (pg/mg tissue) revealed a significant ($p<0.05$) reduction in group 2 (55.45±0.30) when compared to all other groups. In the treatment groups 4, 5 and 6 there was a significant ($p<0.05$) rise in the concentration of IL - 10 when compared to group 2, the relative concentration of IL - 10 was found lowest in the group 5 (61.62±0.36) when compared among the treated groups.

The concentration of TNF- α (pg/mg tissue) revealed a significant ($p<0.05$) rise in group 2 (52.65±3.45) when compared to all other groups. In the treatment groups 4, 5 and 6 there was a significant ($p<0.05$) reduction in the concentration of TNF- α when compared to group 2, the relative concentration of TNF- α was found lowest in the group 6 (27.73±2.34) when compared among the treated groups.

The concentration of NF-κB (pg/mg tissue) revealed a significant ($p<0.05$) rise in group 2 (48.65±2.91) when compared to all other groups. In the treatment groups 4, 5 and 6 there was a significant ($p<0.05$) reduction in the concentration of NF-

κB when compared to group 2, the relative concentration of NF-κB was found lowest in the group 4 (22.37±3.23) when compared among the treated groups.

The concentration of TGF – β (pg/mg tissue) revealed a significant ($p<0.05$) rise in group 2 (529.76±4.20) when compared to all other groups. But the concentration of TGF – β revealed significant ($p<0.05$) decrease in the treated groups as compared to group 2, the relative concentration of TGF – β was found lowest in the group 4(277.66±1.34) when compared among the treated groups.

Histological examination of liver of groups 1and 3 was devoid of abnormalities. Liver exhibited moderate to severe congestion of portal vein (PV) and central vein (CV), narrowing of hepatic cords, dilated sinusoids and pycnotic nuclei of hepatocytes and mild focal fibrosis of periportal area with mild vasculitis and mild vacuolar degeneration of hepatocytes in group 2. In group 4 few binucleated hepatocytes and majority of cells showing normal appearance of hepatocytes with normal nuclei and portal triad, where as in group 5 normal hepatic cords, with mild congestion of central vein (CV) and sinusoids with mild proliferation of Kupffer cells were noticed, But the changes were mild when compared to group 2. In group 6, liver showing mild proliferation of Kupffer cells, mild dilatation of sinusoidal and uniform size hepatocytes, near- to- normal architecture.

Immunohistochemistry of liver sections revealed that treatment with BLF reduced the immunoreactivity for Bcl-2 compared to group 2.

From this study, it is concluded that the NAFLD induced by CCl_4 with HFD can be significantly attenuated by the administration of BLF administered @ 300mg/Kg b.wt than 100mg/Kg b.wt in comparison with simvastatin. The antioxidant and anti-inflammatory properties of BLF reduced secretion of pro-inflammatory cytokines TNF-α and up-regulation of anti-inflammatory cytokines such as IL-10 and TGF- β leading to the suppression of lipid per oxidation and tissue injury.Further BLF also reduced the cholesterol and triglycerides along with reduced activity of ALT.

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