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Bovine Lactoferrin Mitigates Oxidative Stress and Inflammation in CCL4 and High-fat Diet-induced NAFLD in C57BL/6 Mice

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ABSTRACT

Background: Impacting approximately 20-30% of adults, nonalcoholic fatty liver disease (NAFLD) is the leading contributor to liver dysfunction. This condition is characterized by the accumulation of more than 5% fat in the liver without the presence of concurrent chronic liver diseases or alcohol abuse. NAFLD manifests across a spectrum ranging from simple steatosis to more complex forms involving hepatic lesions, inflammation and fibrosis. This research aimed to assess the therapeutic effectiveness of Bovine Lactoferrin (BLF) in a model of NAFLD using male C57BL/6 mice.

Methods: Adult male C57BL6 mice, with an average weight of 28 g, were obtained and maintained in a consistent environment at 22±2°C for 6 weeks, following a regulated 12-hour light-12-hour dark cycle. Control groups 1 and 3 were provided with a standard pellet feed, while model groups 2, 4, 5 and 6 were supplied with a high-fat diet. Induction of NAFLD included administering a high-fat diet (HFD) and CCl4 (0.5 mg/kg with olive oil) twice a week via intraperitoneal injection over 6 weeks. After the experimental procedures, an assessment of serum-biochemical parameters, inflammatory cytokines, anti-oxidant profile and liver histopathology were conducted.

Result: The results revealed significant ($p<0.05$) alteration in sero-biochemical parameters, inflammatory cytokines (IL-10, TNF-α, NF-κB and TGF-β) anti-oxidant 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 compared to disease control. The findings from this study suggest that BLF could potentially function as a therapeutic intervention for mitigating hepatic injury, inflammation and fibrosis in NAFLD by deactivating NF-κB pathway.

Key words: Bovine Lactoferrin, CCL_4, Inflammation, NAFLD, Oxidative stress.

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) affects 20-30% of adults and is characterized by excess fat accumulation in the liver, unrelated to alcohol misuse or other chronic liver conditions (Athyros *et al*., 2017). It ranges from simple fat buildup to more severe forms involving inflammation and fibrosis, increasing the risk of liver diseases like cirrhosis and hepatocellular carcinoma Byrne *et al.* (2016). Recently, experts proposed the term 'metabolic dysfunction-associated fatty liver disease' (MAFLD) to better reflect its underlying mechanisms. Patients with NAFLD might experience conditions like insulin resistance, obesity, dyslipidemia, hypertension, and hyperglycemia (Delhi Bovi *et al.,* 2021). Diagnosis of MAFLD involves detecting liver fat accumulation alongside indicators like obesity, type 2 diabetes, or metabolic dysregulation (Eslam *et al.,* 2020).

Lactoferrin (LF) is a multifunctional protein known for its immunomodulatory, antiviral, anticancer and antimicrobial properties (Cendon *et al.,* 2014). Studies suggest that lactoferrin treatment may improve hematological changes induced by high-fat diet and CCl_4 administration Rao *et al.* (2022). Additionally, it has been shown to positively affect lipid metabolism modulation (Ono *et al*., 2013). However, its potential hypoglycemic and hypolipidemic effects on NAFLD have not been fully elucidated. In this study, we aimed to investigate the effects of dietary LF on NAFLD progression, particularly its hypoglycemic and hypolipidemic effects. We used a NAFLD model induced in C57BL6 mice through a high-fat diet and intraperitoneal injections of carbon tetrachloride (CCl_4) to mimic a metabolic syndrome context.

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MATERIALS AND METHODS

Chemicals

Carbon tetrachloride was sourced from M/s Sigma-Aldrich, St. Louis, MO, USA. Bovine lactoferrin was obtained from Bioven Ingredients, India and Simvastatin from Sun

Pharmaceutical Industries Limited, India. ERBA Diagnostics Ltd, Surat, India, provided standard kits for AST and ALT estimation, while ELISA kits for IL-10, TNF-α, NF-κB and TGF-β were procured from Krishgen Biosystems, Mumbai, India.

Animals and experimental design

Adult male C57BL6 mice weighing approximately 28 g were obtained from VYAS Labs, Hyderabad. A total of 36 mice were housed under controlled environmental conditions for 6 weeks, with a 12-hour light-dark cycle and a temperature of 22±2°C. Control groups 1 and 3 received a standard pellet feed, while model groups 2, 4, 5 and 6 were fed a high-fat diet procured from M/s. VRK Nutritional Solutions, Hyderabad, with unlimited access to water. Approval for the study was granted by the Institutional Animal Ethics Committee in the year 2023 in the Department of Veterinary Pharmacology and Toxicology, College of Veterinary Science, Hyderabad (Approval No. IAEC.MICE/CPCSEA 1/24/C.V.Sc, Hyd, dated 12.06.2021). The following treatment for respective groups is mentioned (Table 1).

Blood and serum analysis

On the experimental day, mice blood was drawn from the retro-orbital plexus using serum vacutainers. Subsequently, the blood samples were centrifuged at 3000 RPM for 15 minutes to separate the serum, which was then stored at -80°C. The stored serum samples were utilized for subsequent analysis of liver biomarkers, including AST and ALT, using the Erba kit IFSS method.

Collection of organs

After the treatment period was completed, the mice were euthanized for 2 minutes using CO_2 chamber with a fill rate of 30-70% of the chamber volume per minute CO_2 and a necropsy was performed to collect liver. Liver tissues were homogenized for anti-oxidant and cytokine analysis. Some tissues were placed in 10% neutral buffered formalin for subsequent histopathological and other staining procedures for evaluation of fibrosis. The liver sections were subjected to histopathological staining (Haematoxylin and Eosin) using the modified methods described by Namratha *et al*. (2019), immunohistochemistry staining procedure as outlined by Renushe *et al*. (2022) and anti-oxidant markers analysed as per procedure adapted in our lab (Kumar and Reddy, 2012; Sneha *et al.,* 2021).

Statistical analysis

The collected data underwent statistical analysis using one-way ANOVA in SPSS version 25.0. Significance was determined with a threshold of $p<0.05$, assessed through Duncan's post hoc test.

RESULTS AND DISCUSSION

Liver serum biomarkers

Groups 2, 4, 5 and 6 showed a significant ($p<0.05$) increase in serum total cholesterol during the 4th and 6th weeks compared to groups 1 and 3. Group 2 exhibited higher cholesterol levels than groups 4, 5 and 6. Triglyceride concentration (mg/dL) significantly ($p<0.05$) rose in groups 2, 4, 5 and 6, while HDL levels (mg/dL) notably ($p<0.05$) decreased during the 4th week compared to groups 1 and 3. By the 6th week, group 2 showed a significant ($p<0.05$) increase in triglycerides and an important ($p<0.05$) decrease in HDL compared to other groups. ALT and AST activity (IU/L) in group 2 significantly ($p<0.05$) increased compared to all other groups, while groups 4, 5 and 6 exhibited a significant ($p<0.05$) decrease in both ALT and AST compared to group 2. Group 3 had comparable levels to group 1, with a significant difference (Table 2).

Anti-oxidant activity

In comparison to all other groups, group 2 demonstrated a notable decrease ($p<0.05$) in the liver's concentration of glutathione (GSH), Catalase and Superoxide dismutase (SOD) activity. Conversely, within the treated groups (4, 5 and 6), group 4 exhibited a substantial ($p<0.05$) elevation in liver GSH concentration, catalase and SOD activity. These findings suggest a distinct impact of the treatments on the anti-oxidant status of the liver, with group 2 experiencing a decrease and group 4 showing an enhancement in key markers in the 6th week. Unlike the other groups, group 2 demonstrated a noticeable rise ($p<0.05$) in the liver's concentration of TBARS and elevated levels of nitric oxide. Among the treated groups (4, 5 and 6), a significant ($p<0.05$) decrease in liver TBARS concentration and nitric oxide levels was observed (Table 2).

Pro-inflammatory and anti-inflammatory activity

Group 2 showed a significant ($p<0.05$) decrease in IL-10 concentration (pg/mg tissue) compared to other groups. In treated groups 4, 5 and 6, there was a significant ($p<0.05$)

Table 1: Experimental design.

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

Table 2: Serobiochemical, anti-oxidant profile and anti-inflammatory parameters in different groups of rats treated with bovine lactoferin against CCL4 and High-Fat Diet-Induced NAFLD in C57BL/6 Mice.

	Sero biochemical parameters					Anti-oxidant profile				Anti-inflammatory and inflammatory markers			
Group	Total cholesterol (mg/dL)	Triglycerides (mg/dL)	HDL-Cholesterol (mg/dL)	ALT levels (IU/L)	AST levels (IU/L)	GSH (nmoles/mg protein)	TBARS (nM of MDA /mg protein)	Catalase activity (U/mg protein)	SOD activity (U/mg protein)	Nitric oxide (µM of nitrite/mg tissue)	IL-10 levels (pg/mg tissue)	TNF- α levels (pg/ mg tissue)	NF-κB levels (pg/ mg tissue)	TGF-β levels tissue)
1	94.50± 1.70^{e}	66.03± 2.37^{e}	52.15± 1.44^{c}	45.23± 1.20^{e}	128.11± 2.11^{e}	24.73± 1.16^{a}	2.19± 0.08^{d}	71.75± 4.80^{b}	6.99± 0.29^{a}	2.75± 0.13^{d}	71.61± 0.42^{a}	26.62± 2.21^{e}	15.82± 4.71^{f}	249.81 ±0.82^{e}
2	203.66± 8.99^{a}	121.95± 11.84^{a}	16.80± 0.67^{e}	244.18± 1.13 ^{a}	264.93± 1.23^{a}	14.98± 0.44^{e}	3.93± 0.04^{a}	43.75± 4.10^{f}	3.47± 0.11^{f}	8.46± 0.72^{a}	55.45± 0.30^{e}	52.65± 3.45^{a}	48.65± 2.91^{a}	529.76 ±4.20^{a}
3	86.78± 3.50^{f}	49.03± 5.99^{f}	59.81± 1.45^{a}	34.03± 1.46^{f}	117.06± 1.48^{f}	24.81± 0.54^{a}	2.09± 0.10^{e}	72.69± 5.92^{a}	6.26± 0.25^{b}	2.58± 0.18^{e}	69.68± 0.33^{b}	24.76± 1.87^{f}	18.46± 4.87^{e}	241.58 ±1.05^{f}
4	116.08± 2.78^{c}	71.06± 7.84^{c}	52.05± 1.50^{c}	84.13± 1.10^{d}	133.90± 1.11^{d}	23.15± 0.67^{b}	2.48± 0.04^{c}	68.91± 3.23^{c}	5.87± 0.05^{c}	3.28± 0.21^{c}	65.54± 0.37^{c}	28.67± 2.23^{c}	22.37± 3.23^{d}	277.66 ±1.34^{d}
5	123.03± 2.89^{b}	73.91± 6.37^{b}	49.21± 1.80^{d}	96.21± 1.19^{c}	139.13± 1.23^{b}	21.24± 0.39^{d}	2.95± 0.06^{b}	65.78± 3.00^{e}	5.12± 0.02^{e}	4.17± 0.16^{b}	61.62± 0.36^{d}	36.87± 2.05^{b}	36.52± 3.95^{b}	313.51 ±1.93^{b}
6	98.31± 3.06^{d}	68.00± 6.85^{d}	55.25± 1.72^{b}	113.03± 1.27 ^{b}	136.10± 1.39 ^{c}	22.91± 0.31^{c}	2.67± 0.02^{c}	67.66± 3.43^{d}	5.74± 0.05^{d}	3.18± 0.17^{c}	64.69± 0.32^{d}	27.73± 2.34^{d}	27.38± 3.74^{c}	279.61 ±1.54^{c}

Means ± S.E with different small alphabets as superscripts differ significantly ($p < 0.05$) among the groups by vertical comparison (n=6).

increase in IL-10 compared to group 2, with group 5 having the lowest IL-10 concentration. Additionally, group 2 exhibited an important ($p<0.05$) rise in TNF-α, NF-κB and TGF-β concentrations (pg/mg tissue) compared to all other groups. Treated groups 4, 5 and 6 showed a significant ($p<0.05$) reduction in TNF-α concentration compared to group 2, with group 6 having the lowest TNF-α concentration and the lowest NF-κB and TGF-β concentration was observed in group 4 (Table 2).

Histopathology of liver

Histological examination of the livers from groups 1 and 3 revealed no abnormalities (Fig 1.1 and 1.3). Group 2 exhibited moderate to severe congestion of the portal vein (PV) and central vein (CV), along with narrowed hepatic cords, dilated sinusoids, pycnotic nuclei of hepatocytes and mild focal fibrosis of the periportal area. Additionally, mild vasculitis and vacuolar degeneration of hepatocytes were observed (Fig 1.2). In contrast, group 4 displayed few binucleated hepatocytes, with the majority showing normal hepatocyte appearance, normal nuclei and portal triad (Fig 1.4). Group 5 exhibited normal hepatic cords, mild congestion of the central vein (CV) and sinusoids and mild proliferation of Kupffer cells, with changes milder than those in group 2 (Fig 1.5). Group 6 showed mild proliferation of Kupffer cells, mild sinusoidal dilatation and uniform-sized hepatocytes, maintaining near-normal architecture (Fig 1.6).

Special Staining: Masson's Trichome Staining (MTS)

Group 1 liver sections displayed a normal architecture with specific staining of the central vein's basement membrane (Fig 2.1). In contrast, group 3 exhibited a similar normal architecture with specific staining (Fig 2.3). In group 2, mild periportal fibrosis and moderate proliferation of fibrous tissue around the portal triad, along with bile duct proliferation and portal vein congestion, were observed (Fig 2.2). Group 4 showed normal liver architecture with very mild fibrous connective tissue proliferation around the portal vein (Fig 2.4). Group 5 displayed moderate fibrous connective tissue proliferation around the bile duct (Fig 2.5), while group 6 maintained a normal architecture with specific staining of the central vein's basement membrane (Fig 2.6).

Special staining: Oil Red O

Group 2 liver sections exhibited mild fatty changes, mild dilated sinusoids and moderate micro and macrovesicular fatty change (Fig 3.1). Conversely, group 3 showed negative fat staining (Fig 3.2). In group 4, liver sections revealed very mild positivity for fat (Fig 3.3), while group 5 displayed moderate fat positivity (Fig 3.4). Group 6 exhibited mild fat positivity (Fig 3.5).

Immunohistochemical analysis

Immunohistochemical analysis of mice liver tissue sections revealed intense cytoplasmic reactivity for Bcl-2 in group 2 (Fig 4.2) compared to group 1 and 3 (Fig 4.1 and 4.3). Groups 4 and 6 exhibited very mild cytoplasmic immunoreactivity for Bcl-2 (Fig 4.4 and 4.6), while group 5

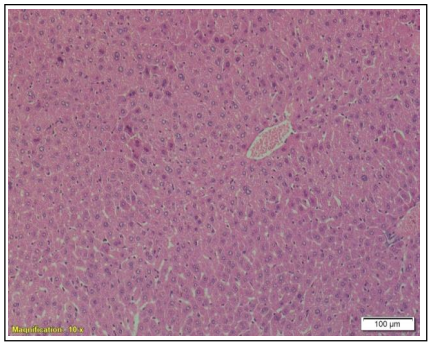

Fig 1.1: Normal architecture of liver (G-1, 10 x).

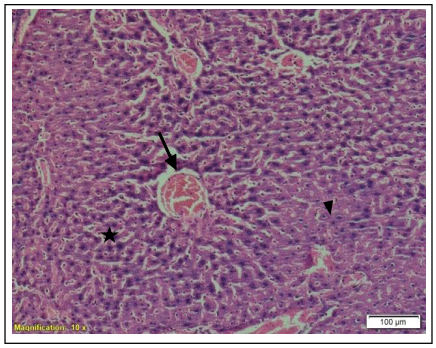

Fig 1.2: Microphotograph of liver showing moderate to severe congestion of portal vein (PV) and central vein (CV), narrowing of hepatic cords, dilated sinusoids and pycnotic nuclei of hepatocytes (G-2, 10 x).

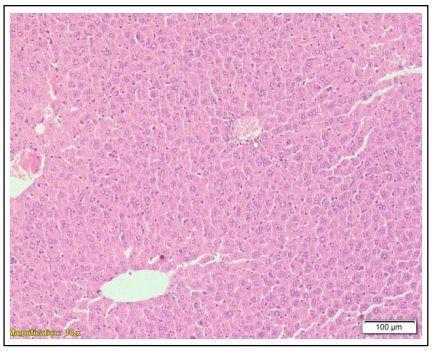

Fig 1.3: Photomicrograph of liver showing Hepatocytes showing uniform hepatic cord with uniform size hepatocytes with normal appearance of Kupffer cells (G-3.10 x).

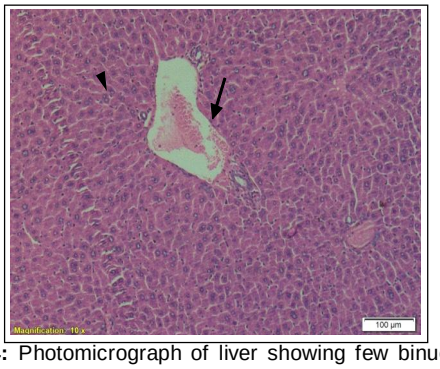

Fig 1.4: Photomicrograph of liver showing few binucleated hepatocytes and majority of cells showing normal appearance of hepatocytes with normal nuclei and portal triad (G4,10 x).

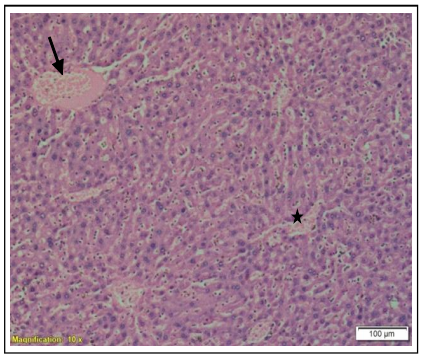

Fig 1.5: Photomicrograph of liver showing normal hepatic cords, with mild congestion of central vein (CV) and sinusoids and mild proliferation of Kupffer cells (G-5, 10 x).

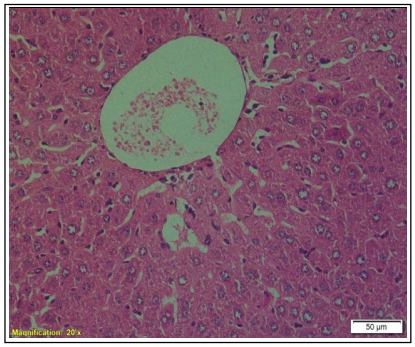

Fig 1.6: Photomicrograph of liver showing mild proliferation of Kupffer cells, mild dilatation of sinusoidal and uniform size hepatocytes (G-6, 20 x).

displayed moderate cytoplasmic immunoreactivity (Fig 4.5).

In the context of metabolic syndrome, nonalcoholic fatty liver disease (NAFLD) represents a significant concern, characterized by steatosis progressing to nonalcoholic steatohepatitis (NASH), fibrosis and cirrhosis (Bugianesi *et al*., 2002). NAFLD is closely linked with metabolic disorders like obesity, insulin resistance (IR) and type 2 diabetes mellitus (T2DM), contributing to its increasing prevalence despite medical advancements (Ishtiaq *et al.,* 2019). Various animal models have been developed to mimic NAFLD, including dietary modifications and chemical induction, providing valuable insights into the pathogenesis of fatty liver and fibrosis in mice and rats.

In our study, we observed the impact of a high-fat diet combined with carbon tetrachloride (HFD+CCl_4) on serum lipid profiles. Notably, we found a significant decrease in high-density lipoprotein (HDL) levels and a marked increase in other lipid parameters in the HFD+CCl_4 group compared

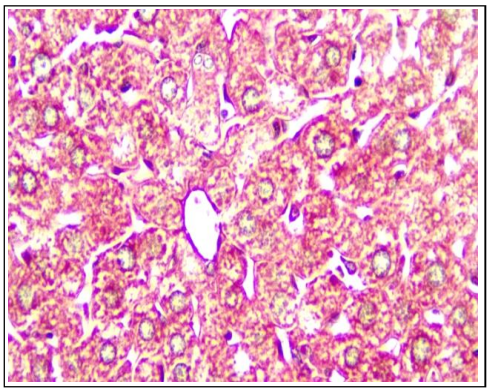

Fig 2.1: Photomicrograph of liver showing normal architecture with specific staining of basement membrane of central vein in group-1 (MTS 400 x).

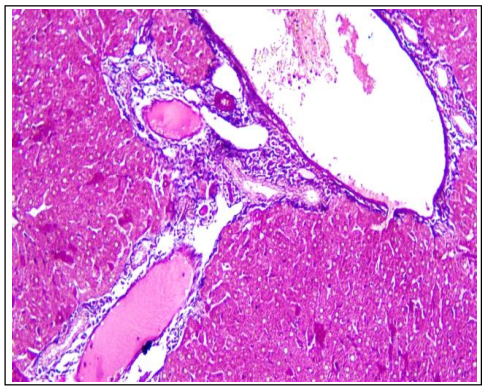

Fig 2.2: Photomicrograph of liver showing moderate proliferation of fibrous tissue around the portal triad along with moderate bile duct proliferation and mild congestion of portal vein in group-2 (MTS 100 x).

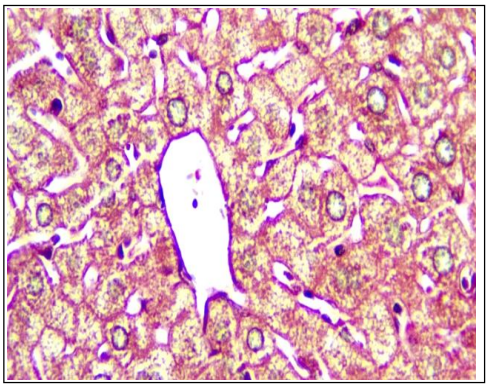

Fig 2.3: Photomicrograph of liver showing normal architecture with specific staining of basement membrane of central vein in group-3 (MTS 400 x).

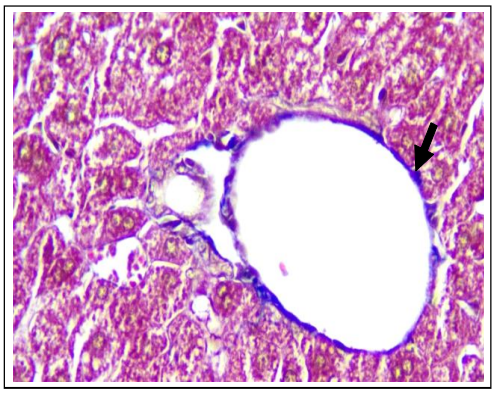

Fig 2.4: Photomicrograph of liver showing very mild proliferation of fibrous connective tissue around the portal vein in group-4 (MTS 400 x).

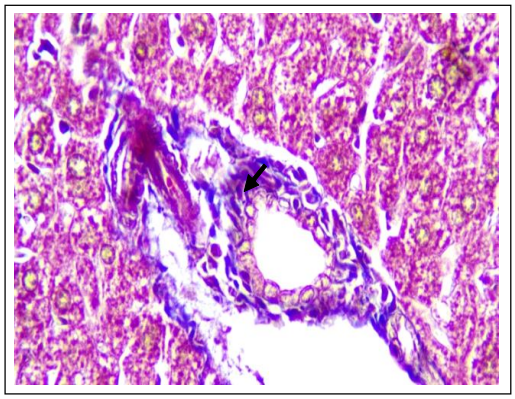

Fig 2.5: Photomicrograph of liver showing moderate proliferation of fibrous connective tissue around the bile duct in group-5 (MTS 400 x).

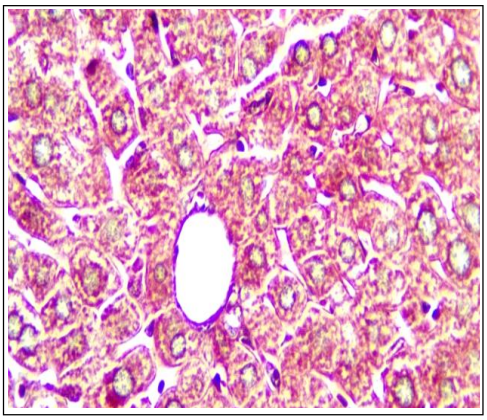

Fig 2.6: Photomicrograph of liver showing normal architecture with specific staining of basement membrane of central vein in group-6 (MTS 400 x).

to control and simvastatin-treated groups. Treatment with bile acid-like compounds (BLF) in certain groups appeared to mitigate these alterations, potentially through enhanced bile acid synthesis and excretion, leading to improved lipid profiles. Our findings are consistent with previous studies suggesting a reduction in cholesterol synthesis and absorption, alongside increased cholesterol excretion, as a mechanism of action for lipid-lowering agents like BLF (Jyothi *et al.,* 2010; Chen *et al.*, 2019).

Moreover, we investigated the impact of BLF treatment on oxidative stress markers and anti-oxidant enzyme activity in the liver. Elevated fatty acid levels are

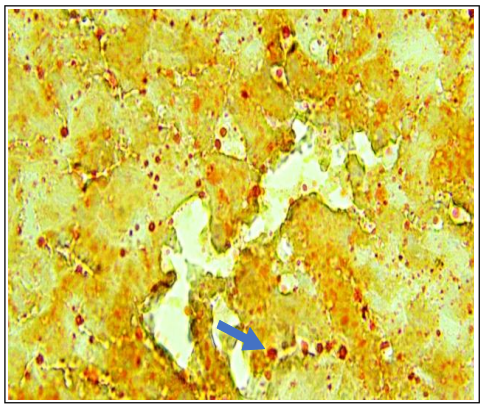

Fig 3.1: Photomicrograph of liver showing moderate micro and macro vesicular fatty change in group-2 (Oil Red O 100x).

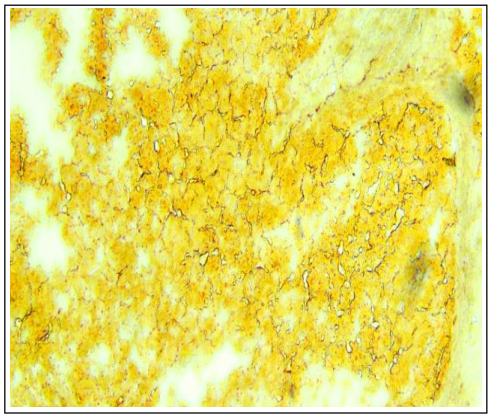

Fig 3.2: Photomicrograph of liver showing negative for fat stain in group-3 (Oil Red O 100x).

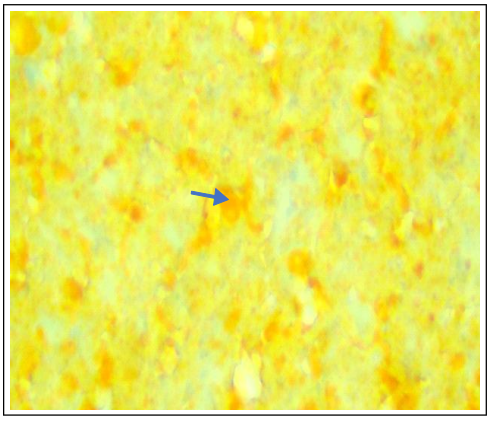

Fig 3.3: Photomicrograph of liver showing very mild positive for fat in group-4 (Oil Red O 400x).

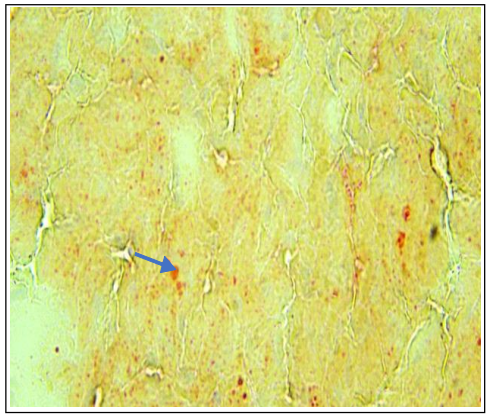

Fig 3.4: Photomicrograph of liver showing moderate positive for fat in group-5 (Oil Red O 100x).

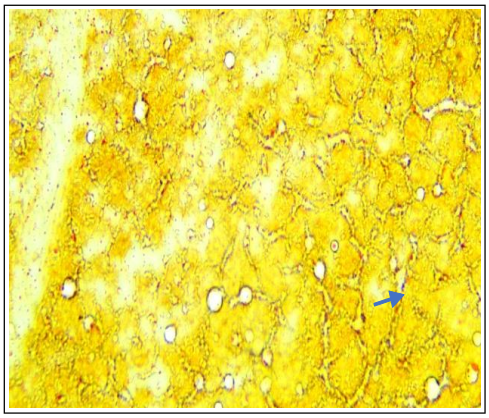

Fig 3.5: Photomicrograph of liver showing mild positive for fat in group-6 (Oil Red O 100x).

known to induce oxidative stress and inflammation, resulting in reactive oxygen species (ROS) formation and hepatocyte damage (Matsunami *et al.,* 2010; Tummala *et al,* 2022 and Vemula *et al.*, 2023). Our results demonstrated a significant reduction in oxidative stress markers and an increase in anti-oxidant enzyme activity in BLF-treated groups compared to the HFD+CCl4 group. These findings support the potential anti-oxidant

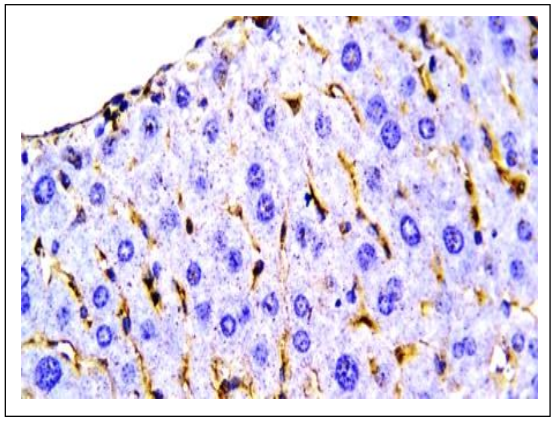

Fig 4.1: Photomicrograph of liver not showing immunoreactivity towards Bcl-2 in group-1 (IHC 400 x).

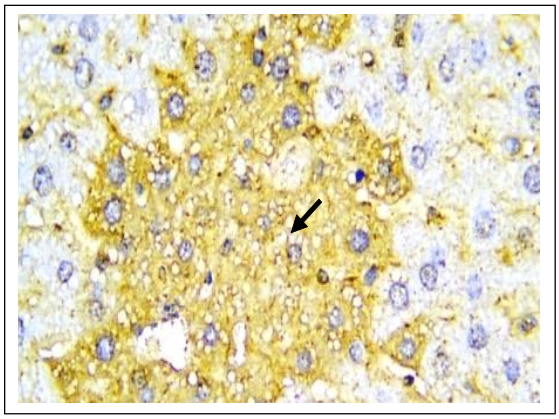

Fig 4.2: Photomicrograph of liver showing intense cytoplasmic reactivity for Bcl-2 in group-2 (IHC 400 x).

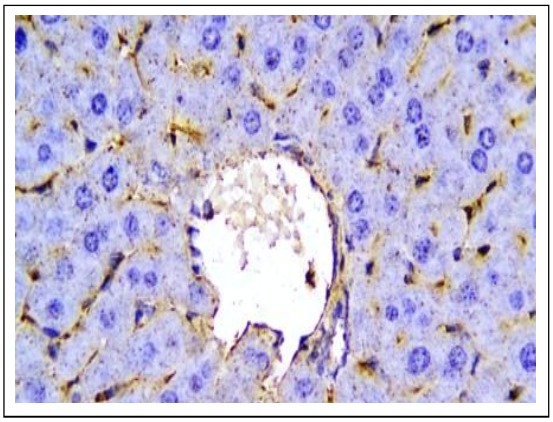

Fig 4.3: Photomicrograph of liver not showing immunoreactivity towards Bcl-2 in group-3 (IHC 400 x).

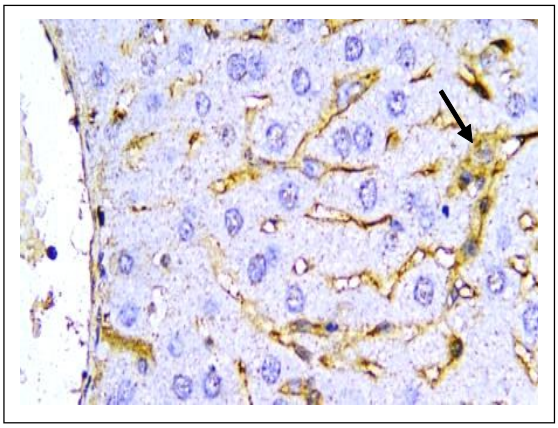

Fig 4.4: Photomicrograph of liver showing very mild cytoplasmic immunoreactivity for Bcl-2 in group-4 (IHC 400 x).

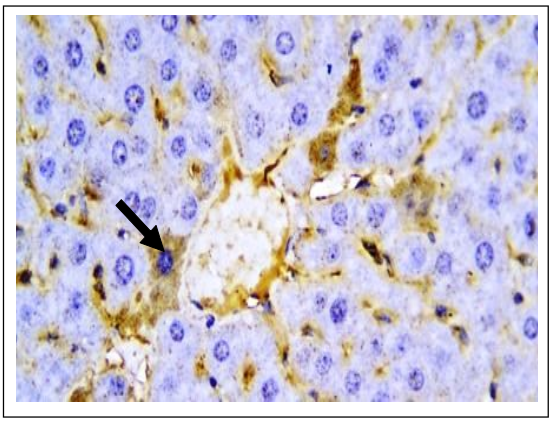

Fig 4.5: Photomicrograph of liver showing moderate cytoplasmic immunoreactivity for Bcl-2 in group-5 (IHC 400 x).

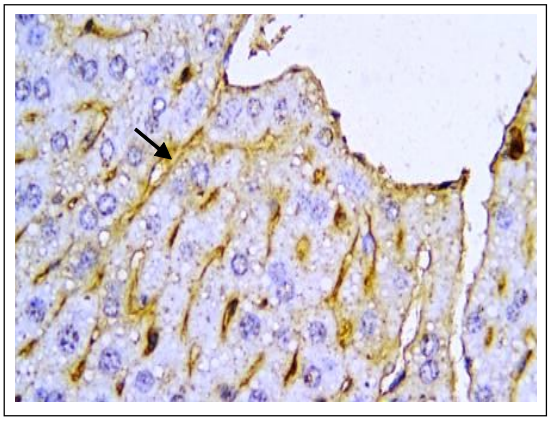

Fig 4.6: Photomicrograph of liver showing very mild cytoplasmic immunoreactivity for Bcl-2 in group-6 (IHC 400 x).

properties of BLF, possibly through regulation of redox cycling and hydroperoxide decomposition (Chen *et al*., 2016).

Furthermore, we evaluated pro-inflammatory cytokine levels and histopathological changes in the liver. The HFD+CCl_4 group exhibited elevated levels of pro-inflammatory cytokines, indicative of inflammation and oxidative stress. Treatment with BLF attenuated these inflammatory responses, leading to histopathological improvements such as reduced congestion, sinusoidal dilatation and hepatocyte damage. Our results are consistent with previous

studies reporting similar anti-inflammatory effects of BLF (Farid *et al.,* 2021; Naiki-Ito *et al.*, 2020).

Overall, our findings highlight the potential therapeutic effects of BLF in mitigating NAFLD-associated metabolic and inflammatory changes. Further research is warranted to elucidate the underlying mechanisms and optimize the use of BLF as a potential treatment option for NAFLD and associated metabolic disorders.

CONCLUSION

The study highlights significant alterations in liver serum biomarkers among different treatment groups. Groups 2, 4, 5 and 6 exhibited noteworthy changes in total cholesterol, triglycerides, HDL, ALT and AST levels along with a considerable decrease in anti-oxidant parameters, pro-inflammatory cytokines (TNF-α, NF-κB, TGF-β and IL-10) and histopatholgy. Particularly, group 2 demonstrated a distinct elevation in total cholesterol, triglycerides and liver enzyme activities compared to other groups. Conversely, groups 4, 5 and 6 displayed mitigated effects, suggesting potential protective roles of the administered treatments. In conclusion, the study underscores the diverse effects of treatments on liver biomarkers, anti-oxidant status, inflammatory responses, histopathology and apoptotic regulation. These findings contribute valuable insights into the potential therapeutic efficacy of the administered treatments in mitigating liver-related conditions. Further research is warranted to elucidate the underlying mechanisms and optimize treatment strategies.

Conflict of interest

All authors declared that there was no conflict of interest.

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