Inhibition of NF-κB and Cox-2 by Lirioresinol B Dimethyl Ether in DEN induced Hepatic Fibrosis Mouse Model

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And my success cannot come from any source besides Allah. (Surah Hud 11:88)

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Abstract

Cancer is a major public health problem and the second leading cause of mortality around the world. Hepatocellular carcinoma (HCC) is one of the most common cancers, associated with high mortality. Almost 80% of HCC cases are associated with chronic hepatitis and cirrhosis resulting from inflammation and fibrosis. Therefore, novel therapeutic agents with multifactorial effects are urgently needed for the treatment of chronic hepatitis and prevention of other hepatic diseases.

Lirioresinol B Dimethyl ether (LBDE) is a bioactive phytochemical obtained from seed oil of *Magnolia Fargesii*, with anti-inflammatory activity. In this study, Balb/c mice were used to induce hepatic fibrosis by administration of diethyl nitrosamine (DEN) and carbon tetra chloride (CCl4) for a period of 10 weeks, followed by treatment with LBDE and 5-flourouracil (5-FU, as a positive control).

The histopathological examination revealed that LBDE treatment recovered fibrosis was at week 14, while mice treated with only DEN/CCl4 had severe fibrotic liver. Furthermore, the serum levels of alanine aminotransferase (ALT), alkaline phosphatase (ALP) in serum significantly increased in DEN/CCl4 model group as compared with the control. The serum levels of LBDE and 5-FU were decreased and were more towards the normal range. Treatment with 5-FU showed a significant decrease in the lipid peroxidation as confirmed by decrease concentration of malondialdehyde and increase in antioxidant enzymes such as super oxide dismutase and reduced glutathione. Increased level of antioxidants in liver of LBDE and 5-FU treated group indicated the healing of hepatocytes as compared to DEN/CCL4 group. The immunoblot analysis revealed inhibition of COX-2 and NFκB by LBDE as indicated by western blot analysis. Our result confirmed the potential therapeutic role of LBDE for the treatment and prevention of hepatic diseases

Key Words:

Hepatic fibrosis, DEN+CCL4, Lirioresinol B Dimethyl ether, 5-flourouracil

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List of abbreviation

CCL 4	Carbon tetra Chloride
COX-2	Cyclooxygenase-2
DEN	Di-ethyl Nitrosamine
DPPH	1, 1-Diphenyl-2-picryl-hydrazyl
BSA	Bovine Serum Albumin
5-FU	5-Flouro Uracil
HCC	Hepatocellular Carcinoma
LBDE	Lirioresinol B Dimethyl Ether
NC	Nitrocellulose
NF-κB	Nuclear Factor Kappa B
PBS	Phosphate Buffer Saline
SDS	Sodium Dodecyl Sulfate
SOD	Super Oxide Dismutase
TCA	trichloroacetic acid
TBA	Thiobarbituric acid
TBAR	Thio-Barbituric Acid Reactive Substances
RANKL	Receptor Activator of NF-κB Ligand
UV-Vis	Ultra Violet Visible

1 INTRODUCTION

Liver plays its role as the most vital organ responsible for variety of chemical reactions necessary for survival. It is classified as gland associated with bile production, metabolism of carbohydrates, fats and proteins, blood filtration and several other functions (Newman, 2018). Injury to liver can be caused by a variety of factors, fibrosis is a healing effect of these injuries that can leads to cirrhosis and eventually to hepatocellular carcinoma. There are multiple risk factors for liver fibrosis including alcohol abuse, genetic abnormalities, viral hepatic infections (HBV, HCV), obesity, metabolic syndrome (Blachier, Leleu, Peck-Radosavljevic, Valla, & Roudot-Thoraval, 2013) .

The fundamental element of liver fibrosis leading to hepatitis induced by variety of conditions such as autoimmune hepatitis, alcohol hepatitis viral hepatitis, is oxidative stress (Wasmuth, Lammert, & Matern, 2003). The severity of oxidative stress determines the progression to fibrosis, a less intense oxidative stress can withstand progression of fibrosis through activation and changes in the hepatic stellate cells. This also include the promotion of proliferative activity, the synthesis and remodeling of the extra cellular matrix, proinflammatory activity, loss of retinoid, contractility, chemotaxis (Kershenobich Stalnikowitz & Weissbrod, 2003). 0.1% of populations according to the European union is affected by liver cirrhosis, annually causing 170,000 deaths (Blachier et al., 2013). The epidemiological evidence shows that liver fibrosis and cirrhosis beside Hepatic carcinoma, imply a great economic load on the society. And for the matter of fact when the traditional methods for treatment fails, the only option left for the decompensated cirrhosis is hepatic transplantation (Pedersen, Bendtsen, & Møller, 2015). In Europe there is a massive increase in the rate of liver transplantation with each passing year estimated up to €100,000 the initial year and €10,000 annually thereafter.(van Agthoven et al., 2001).

Therefore, its clear need of new therapeutics for the treatment of liver disease is an urgent need of today's world.

Plant and their compounds are in use from the ancient times and their medicinal properties are evident in the work of Domitrović & Jakovac, 2011; Hussain, Siddiqui, Fareed, Vijayakumar, & Rao, 2012; Liu et al., 2006 and others. In the regions of North America and Asia Magnoliaceae family and other members of this family are in use from centuries, their products are extensively

used in herbal medicines. The genus magnolia and many of its taxa are the producer of compounds with significant invitro bioactivities, the bioactive compounds include terpenoids, lignans and alkaloids.(Schühly, Khan, Nikolaus, & Fischer, 2001).

Lirioresinol B Dimethyl Ether is a bioactive chemical compound, obtained from seed oil of Magnolia Fargesii, Family of Magnolieceae. Magnolia Fargesii is found in Japan, Korea and China. It is being used in Korean medicines from a long time. Seed oil of the Flower Magnolia Fargesii is rich in phytochemicals like lignans, terpenoids, polyphenols, and alkaloids. Pinoresinol Dimethyl Ether, Fargesin, magolin, aschantin, demethoxyaschantin, fargesin, magnolin, pinoresinol dimethyl ether, liroresinol-B, phillygenin, epimagnolin, de-O methylmagnolin, pinoresinol, eudesmin and epimagnolin A, Lirioresinol B Dimethyl Ether are the lignan compounds reported to be present in seed oil of Magnolia fargessi buds with potential anti- microbial, anti-depressant, anti-rhinitis and various other pharmacological benefits. Lirioresinol B Dimethyl Ether is one of the lignan phytochemicals possessing 6 methoxyl groups, 8 aliphatic protons and 4 aromatic protons (Song & Fischer. H, 1999; Kakisawa, Chen, & Hsü, 1972) .

Lirioresinol B Dimethyl Ether is anti-inflammatory phytochemical. Inflammatory cytokines when stimulate osteoclastic differentiation, receptor activator of NF-κB ligand (RANKL) is up regulated, thus boosting inflammatory pathway. Lirioresinol B Dimethyl Ether possessing anti-inflammatory activity reduces this RANKL induced osteoclastic activity (Jun et al., 2012). Along with other lignans extracted from Magnolia Flos are collectively known as ‘mammalian lignans’ owing to their potential as phytoestrogens. It is one of the three active constituents of MF 5 extracts responsible for inhibiting ovariectomy stimulated osteoporosis progression. Breast Cancer is also known to stimulate bone destruction by hormonal imbalance and cancer metastasis. Because of Breast cancer, inflammatory cytokines like RANKL are stimulated and in turn promote osteoclastic bone destruction. This bone destruction further induces growth factors thus promoting tumor growth. In this way, positive feedback loop keeps promoting bone destruction and tumor growth. This vicious cycle is reported to be blocked by anti-inflammatory phytochemicals like MF extracted lignans. One of the potential anti-inflammatory lignans is Lirioresinol B Dimethyl Ether that is reported to potentially inhibit bone destruction and cancer progression by blocking vicious cycle (Jun et al., 2014).

This study was conducted to investigate potential role of plant isolated compound Lirioresinol B Dimethyl Ether in cancer prevention and treatment of fibrotic liver.

Liver fibrosis is the most common type of liver disease which can be caused by a wide group of factors including alcohol abuse, genetic abnormalities, viral hepatic infections (HBV, HCV), obesity, metabolic syndrome the significant feature of which is the accumulation of extra-cellular matrix due to elevated production and deposition of connective tissue and or lowered or disturbed degradation of the extracellular matrix (S. L. Friedman, Maher, & Bissell, 2000; Wells, 2005). The Underlying pathways for chronic hepatitis are known to be associated with progression from a fibrotic liver to cirrhosis ultimately leading tot the final stage of hepatocellular carcinoma. Anti-inflammatory agents are well known to be used for various conditions including both acute and chronic, therefore, the anti-inflammatory phytochemical Lirioresinol B Dimethyl ether has been used for the treatment of fibrotic liver to exclusively prevent cancerous condition.

In this study, Lirioresinol B Dimethyl Ether was identified as an agent which could be used for the treatment of fibrotic liver accordingly showing its efficacy against cancer initiation and progression. There is very less literature available for the use of LBDE in the past especially against any type of liver condition. But it showed potential anti-inflammatory activity by interfering in the pathway of NF-κB and Cyclooxygenase-2 (COX-2). NF-κB is a key regulator of inflammatory transcription factor, responsible for transcription of inflammatory genes also Cox-2 ia an enzyme that is responsible for the production of prostaglandin which ultimatelty cause inflammation. Both these NF-κB and Cox-2 help in promoting cell survival, cell proliferation and anti-apoptotic protein production Suppression of NF-κB and blockage of COX-2, promote downregulation of various inflammatory cytokines ultimately blocking inflammatory pathways and preventing the liver from fibrosis. In addition to this Lirioresinol B Dimethyl Ether was found to up-regulate inhibitory protein I-κB, which is responsible for inhibition of NF-κB nuclear translocation. Onset of inflammation induces inflammatory cytokines that stimulate enzymes for phosphorylation and proteosomal destruction followed by ubiquitination of I-κB. This step ends with release of NF-kb and its nuclear translocation to promote transcription of inflammatory genes like COX-2, MMPs and TGF-alpha. But I-κB when up-regulated through inhibition of its phosphorylation by a phytochemical, it does not allow nuclear translocation of NF-κB. Protein expression analysis of NF-κB and I-κB was done by Western Blot analysis.

These protein expressions were then compared among different groups (control, diseased and treated) used for experimentation purpose. Data strongly supported anti-inflammatory and anti-cancer activity of Lirioresinol B Dimethyl Ether. For a substance to exhibit anti-inflammatory activity, it is also observed that it possess antioxidant activity as well. For this, Free Radical Scavenging activity of Lirioresinol B Dimethyl ether was observed by using DPPH assay. This assay was performed in a way that it not only calculated anti-oxidant activity of a phytochemical but also compared anti-oxidant activity of LBDE at different concentrations. Increasing concentration of LBDE showed increased antioxidant activity. Furthermore other biochemical assays including reduced glutathione assay, thio-barbituric reactive acid substance assay and super oxide dismutase assay were performed for the evaluation of antioxidant properties of LBDE.

2 LITERATURE REVIEW

2.1 Hepatotoxins

Hanatoxins are the chemical substances that are damaging to the liver tissue, they have the tendency to cause distortion to the liver tissue. In the body they can be present as a side effect of drugs or other factors (Waring et al., 2001). Di-ethyl nitrosamine (DEN) and Carbon-tetrachloride (CCl4) are the toxins used in this study.

2.1.1 Di-ethyl nitrosamine (DEN)

Di-ethyl nitrosamine (DEN) is frequently used in research as a potential carcinogenic regent, its action is specie specific, it has been used for the induction of liver fibrosis cirrhosis in mice and other diseases in mice such as respiratory tumors, gastro-intestinal, hematopoietic and skin tumors (Wang et al. 1992; Binato et al. 2008; Gray et al. 1991). At a cellular scale they perform the biotransformation, as a result of which reactive oxygen species (ROS) are produced in a huge amount, all together they react with the cellular content i-e nucleic aci proteins and lipids causing the malfunctioning of the cell and promoting the progression of centrilobular necrosis.(Aparicio-Bautista et al., 2013; Binato et al., 2008b; Sánchez-Pérez et al., 2005)

2.1.2 Tetra-Chloromethane

Tetra-Chloromethane or Carbon-tetrachloride (CCl4) is a compound that is in use from decades for the induction of oxidative stress. For CCl4 its presumed that it generates the reactive oxygen species facilitated peroxidation of lipids proceeding to the lipid derived oxidative products accumulation which ultimately leads to the liver injury (Poli, Albano, & Dianzani, 1987) and furthermore the deposition of the excessive collagen in the hepatic tissue will leads to the fibrotic condition. During the past decades several studies have been performed demonstrating that the effect of CCl4 induced toxicity predominantly the hepatotoxicity can be prevented by antioxidant compounds, by the inhibition of peroxidation of lipids suppressing the levels of liver function biomarkers whose level elevates during hepatic injury and also by increasing the concentration and rate of antioxidants enzymes activity (Domitrović & Jakovac, 2011; S. L. Friedman et al., 2000; Hussain et al., 2012; Liu et al., 2006; Uehara et al., 2013)

2.2 Phytochemicals

Phytochemical is word that is derived from the Greek linguistics, meaning plant derived chemicals, these the the plant components of plants having non-nutritive properties. These compounds processes the disease preventing protective properties, from the ancient times phytochemicals have been linked with the prevention and treatment of chronic diseases namely, cardiovascular diseases, verity of malignancies, diabetes, hypertension and other wide range of conditions associated with the malfunctioning of the body (Surh, 2003).

2.3 Lirioresinol B Di-methyl Ether

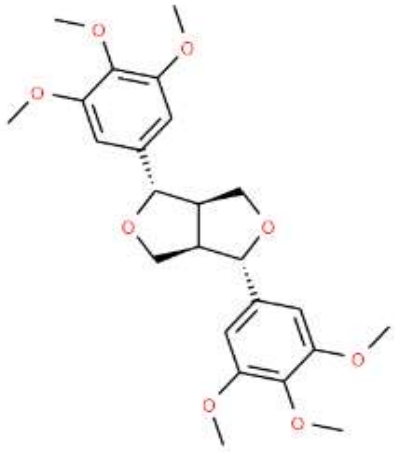

Figure 2.1 Lirioresinol B Dimethyl Ether

Lirioresinol B Dimethyl Ether is a bioactive chemical compound. It is obtained from Magnolia Fargesii (Family Magnolieceae) buds (Kakisawa, Chen, & Hsü, 1972b). Magnolia Fargesii tree is native to china and it grows almost 20m in height and possess broad grean leaves. Seed oil of the Flower Magnolia Fargesii is rich in phytochemicals like lignans, terpenoids, polyphenols, and alkaloids. Lirioresinol B Dimethyl Ether is one of the lignin phytochemicals (Song & Fischer. H, 1999). This phytochemical has been found to possess 6 methoxyl groups, 8 aliphatic protons and 4 aromatic protons (Kakisawa et al., 1972b). Structural formula is shown in figure 1. Owing this Structural formula, LBDE possess medicinal properties. LBDE is known to have potential effectiveness against rhinitis, microbial infection and inflammation (Shen et al., 2008).

2.3.1 Pharmacological Potential of Lirioresinol B Dimethyl Ether:

Lirioresinol B Dimethyl Ether along with other lignans extracted from Magnoli Flos are collectively known as 'mammalian lignans owing to their potential as phytoestrogens. It is one of the three active constituents of MF extracts responsible for inhibiting ovary-ectomy stimulated osteoporosis. Inflammatory cytokines when stimulate osteoclastic differentiation, receptor activator of NF-κB ligand (RANKL) is up regulated, thus boosting inflammatory pathway. Lirioresinol B Dimethyl Ether possessing anti-inflammatory activity reduces this RANKL induced osteoclastic activity (Jun et al., 2012). Breast Cancer is also known to stimulate bone destruction by hormonal imbalance and cancer metastasis. Because of Breast cancer, inflammatory cytokines like receptor activator of NF-κB ligand (RANKL) are stimulated and in turn promote osteoclastic bone destruction. This bone destruction further induces growth factors thus promoting tumor growth. In this way, positive feedback loop keeps promoting bone destruction and tumor growth. This vicious cycle is reported to be blocked by anti-inflammatory phytochemicals like MF extracted lignans. One of the potential anti-inflammatory lignans is Lirioresinol B Dimethyl Ether that is reported to potentially inhibit bone destruction and cancer progression by blocking vicious cycle (Jun et al., 2014).

2.3.2 Properties of LBDE

Table 2.1 Properties of LBDE

S.no	Lirioresinol B Dimethyl Ether	Properties
1	Molecular weight	446.496 g/mol
2	Molecular formula	C24H30O8
3	Structural properties	6 methoxyl groups, 8 aliphatic protons and 4 aromatic protons
4	Solvent	Dimethyl Sulfoxide DMSO

2.4 Fibrosis

Liver fibrosis is the most common type of liver disease which can be caused by a wide group of factors including alcohol abuse, genetic abnormalities, viral hepatic infections (HBV, HCV), obesity, metabolic syndrome the significant feature of which is the accumulation of extra-cellular matrix due to elevated production and deposition of connective tissue and or lowered or disturbed degradation of the extracellular matrix (S. L. Friedman et al., 2000; Wells, 2005).

The Underlying pathways for chronic hepatitis are known to be associated with progression from a fibrotic liver to cirrhosis ultimately leading to the final stage of hepatocellular carcinoma fig2.

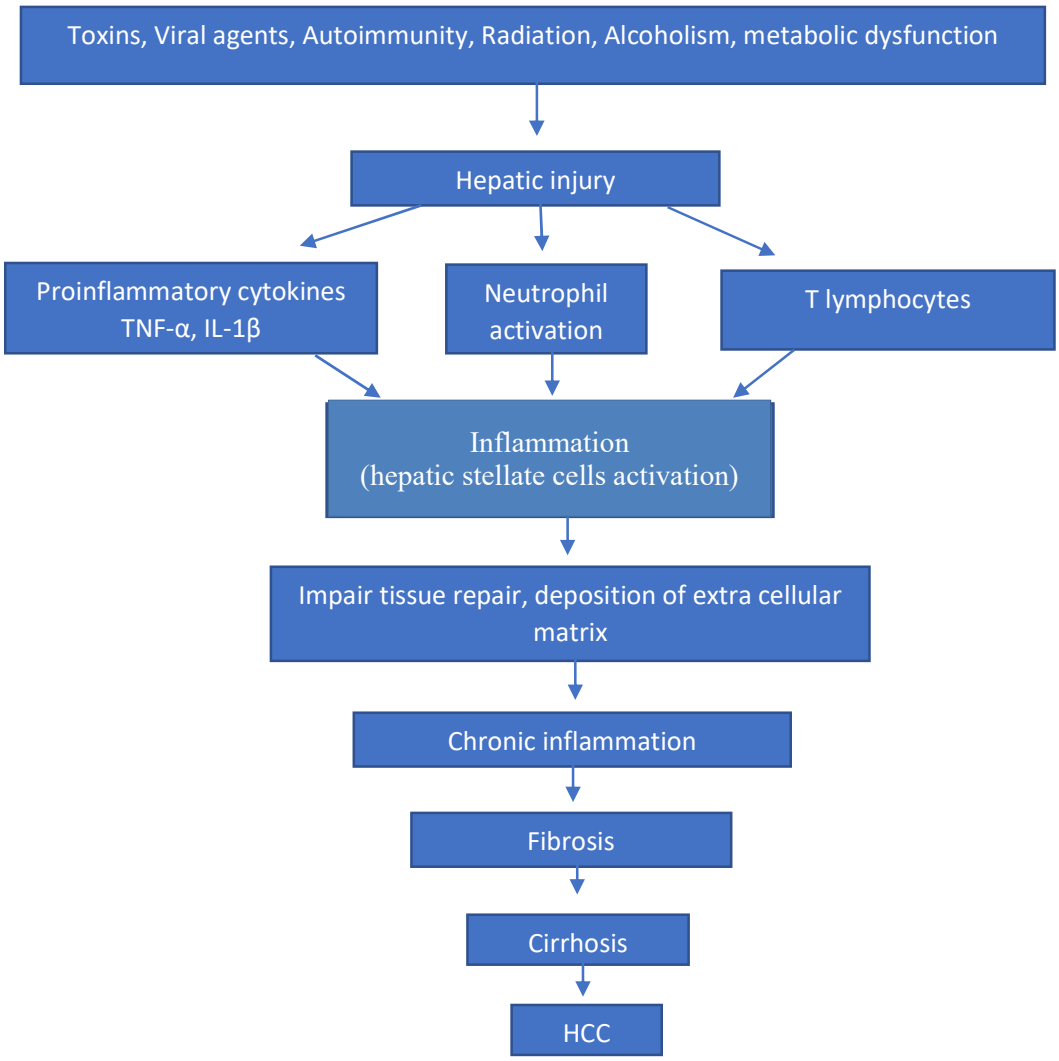

Figure 2.2 Mechanism for the progression of liver injury induced by the associated risk factors contributing to hepatic fibrosis.

After the injury to the liver, it starts its repair system which includes inflammatory changes resulting in the proliferation of the extra cellular matrix (ECM) forming cells and the presence of uninuclear inflammatory modulators (Wick et al., 2010).

2.4.1 Mechanism of fibrosis

Normally the fibrosis tissue in the liver is only restricted to the area of hepatic portal tracts, containing the bile duct and the hepatic artery. As described earlier the general response to the hepatic injury is the activation of inflammation where the hepatic stellate cells are activated that leads its way to the events where the destruction and remodeling of the ECM occur as shown in the figure 1, inflammatory cytokines are produces that is interleukins. TGF-β, IG-1, in the liver the acute phase proteins are produced i-e α2-macroglobulin and the haptoglobin along with the increased level of liver function biomarkers Alanine transaminase and Aspartate transaminase.(S. Friedman, 2003). The continues activation of the hepatic stellate cells leads to the excessive production of the extracellular matrix. Around the hepatocytes the peri sinusoidal tissue also increases, along the way of progression of the disease the fibrous septa creates bridges among the portal tract in the hepatic tissue. As all these events happens, the architectural distortion continues with the expansion of the septa, the distinguishing cirrhosis nodules are formed (Cowan, Rahman, & Krishna, 2010).

2.5 Inflammation-fibrosis-cancer axis

The epidemiological analysis shows that the incident of hepatitis is increasing with its full zeal and zest not only in Europe and ameriica but also in the esteren reagions of the world for example china and japan. Its increase in mainly related with the increasing level of obesity as the its incidence in the last few decades is associated with the natural history of obesity and also to the other factors of nonalcoholic fatty liver diseases. 90% of the hepatic carcinomas are associated with the untreated liver inflammation and fibrosis or in some cases cirrhosis regardless of the risk factor involved in causing the disease at the very first place. Therefore, the approaches in the approaches in prevention of HCC should be entirely focused on inhibiting the underlying molecular mechanisms of inflammation and fibrosis figure 3 (Elsharkawy & Mann, 2007).

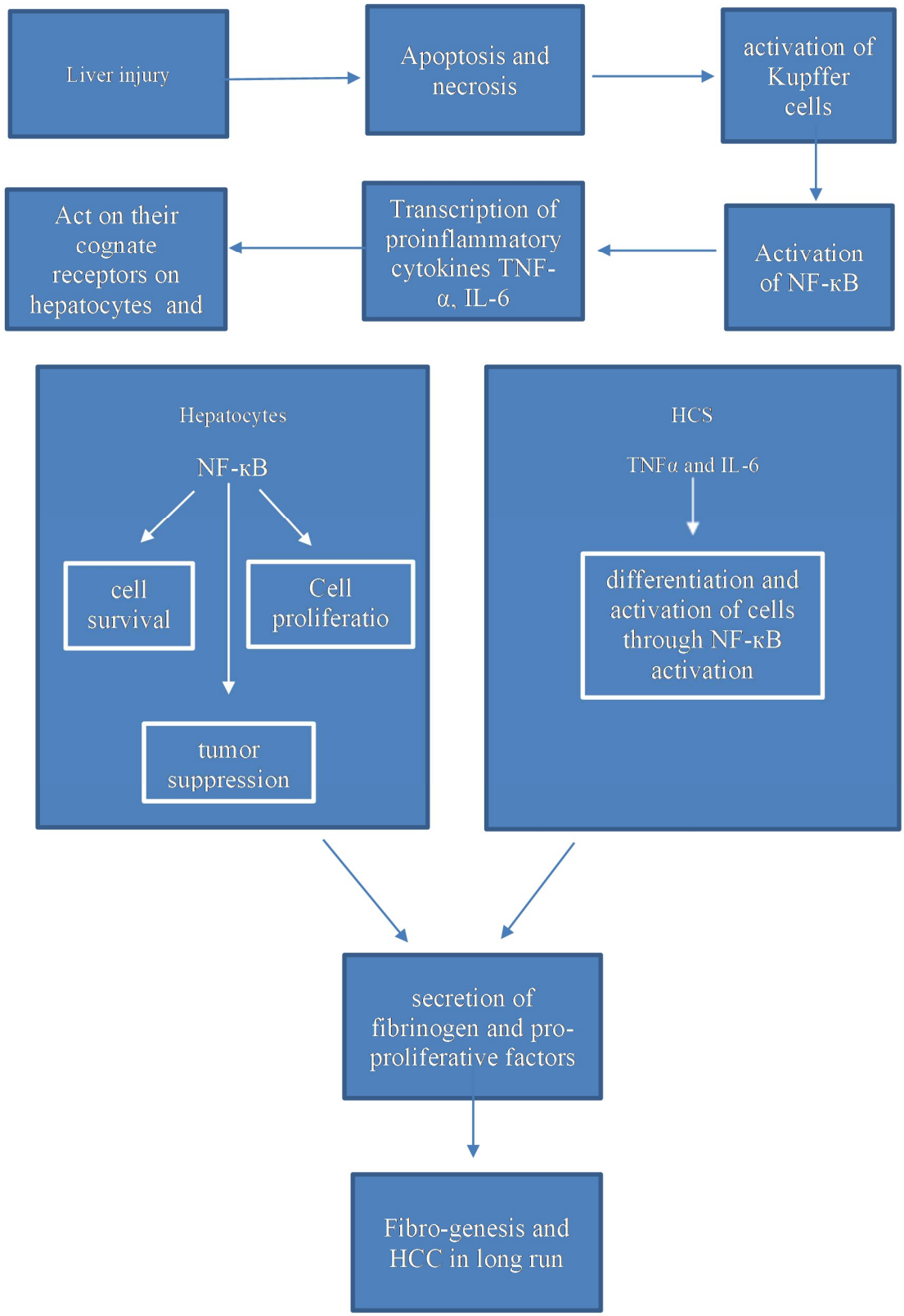

Figure 2.3 Inflammation cancer axis

2.6 Molecular Mechanisms:

Evidences show that oncogenes of tumors might stimulate inflammatory cascade by inducing expression of inflammatory cytokines like IL-1β, IL-6, matrix metalloproteinase (MMPs), Cyclooxygenases (COX-2), pro-apoptotic proteins and angiogenesis promoting proteins (Spitzer, Zheng, Kolls, Vande Stouwe, & Spitzer, 2002).

Key inflammatory regulator, a transcription factor NF-κB is activated by TNFα in liver inflammation figure 3. In studies conducted to understand complex role of NF-κB as a transcription factor, genetic transformation I-κB and inactivation of NF-κB is done to induce HCC, thus showing role of NF-κB in liver fibrosis that subsequently develops into HCC (Elsharkawy & Mann, 2007).

2.7 NF-κB/I-κB and Liver Injury

NF-κB is known to regulate physiology and pathology of liver. Injury to the liver cells simulate pre-apoptotic cascade through caspase activation and at the same time, survival pathway is also turned on by NF-κB activation. NF-κB belongs to Rel Family and it has 5 subunits namely p50 (NF-kB1), p52 (NF-kB2), c-Rel (Rel), p65 (RelA), and RelB (Elsharkawy & Mann, 2007). NF-κB possess N terminal domain, which is responsible for binding DNA, and interaction with I-κB (inhibitory protein) and nuclear translocation of NF-κB (Ghosh & Karin 2002). NF-κB activation is dependent upon phosphorylation of I-κB followed by second step in which I-κB is ubiquitinated and degraded by proteasome. After phosphorylation of I-κB, NF-κB is released in a way that it can freely translocate into nucleus and being transcription factor, activate transcription of inflammation promoting genes e.g., TNF-alpha, COX-2, MMPs etc. (Yamamoto & Gaynor, 2004a) (Karin, 1999). As mentioned above, when chronic inflammation establishes, a vicious cycle is started. Inflammatory genes activated by NF-κB further enhance inflammation and continue to activate enzymes responsible for I-κB phosphorylation and degradation followed by NF-κB nuclear translocation (Luedde & Schwabe, 2011).

2.8 COX-2 and liver injury

Cyclooxygenase-2 COX-2 is an enzyme that catalyzes Prostaglandin (PG) and act as the mediator of the prostaglandin's pathway. In this pathway the arachidonic acid (AA) is converted

to PG and the reaction is catalyzed by the enzymatic activity of COX-1 and COX-2. COX-1 responsible for homeostasis as they produce prostaglandins which are involved performing homeostatic functions (Crofford, 1997) while Cox-2 mainly produce prostaglandins that play effective role in promoting inflammation (Rouzer & Marnett, 2009). It has been reported that various growth factors such as platelets derived growth factors (PDGF), epidermal growth factor (EGF), along with thrombin, they all activates/ stimulates COX-2 pathway (S. L. Friedman et al., 2000).

COX-2 over-expression has been reported in chronic hepatitis, cirrhosis and human HCC in experimentally developed HCC models. Prostaglandin E2 expression is also increased in cancerous hepatocytes and play role in tumor progression, angiogenesis, proliferation and survival. COX-2 inhibitors have inhibitory effect on chronic hepatitis and Hepatocellular Carcinoma (Wu, 2006). Angio-genic factors, adhesion molecules and most importantly COX-2 enzyme are also induced by NF-κB signaling pathway and they all work in a loop activating one another (Costa, Scholer-Dahirel, & Mechta-Grigoriou, 2014).

2.9 Progression to Cancerous Condition:

Some studies have concluded that, growth factors like EFGR expressed in hepatocytes are stimulated by inflammatory cytokines e.g., TNFα stimulated TGFα and COX-2 stimulated AR in mice hepatocytes (Berasain et al., 2005). Molecular mechanisms recently proposed for inflammatory pathways also report that, TGFβ a growth factor like TGFα, is found to be produced by non-parenchymal hepatocytes in inflammation (Gressner, 1995). It stimulates activation of TNFα via NF-κB signaling pathway (Murillo et al., 2007). In addition to this, inflammatory cytokines also induce proteolytic release of growth factors from hepatocytes. This release is controlled by MMPs and promotes inflammatory signaling cascade. This action of MMPs is up-regulated in chronic hepatitis. Extracellular shedding of AR and EGFR through cellular membranes promote hepatocyte survival and proliferation(Drucker et al., 2005). Above mentioned signaling interactions indicate frequent cross-talk among various inflammatory pathways. Most accurately described is that of COX-2 catalyzed prostaglandins pathway. COX-2 over-expression has been reported in chronic hepatitis, cirrhosis and human HCC in experimentally developed HCC models. Prostaglandin E2 expression is also increased in cancerous hepatocytes and play role in tumor progression, angiogenesis, proliferation and

survival. COX-2 inhibitors have inhibitory effect on chronic hepatitis and Hepatocellular Carcinoma (Wu, 2006). All the above-mentioned inflammatory cytokines are induced by key inflammatory regulator NF-κB. Angio-genic factors, adhesion molecules and COX-2 enzyme are also induced by NF-κB signaling pathway. NF-κB is also known to promote cell survival by inducing anti-apoptotic genes like such as BCL-2 and thus initiates and up regulates cancer progression pathways (Greten et al., 2004).

3 METHODOLOGY

The methodology is divided into to 2 distinct categories the in-vivo study and the invitro study. Before the beginning of the invivo study free radical scavenging activity of LBDE was performed to understand and evaluate the free radical scavenging activity of LBDE.

3.1 Materials and instruments

DEN, CCL4, 5-FU, Olive oil, methanol, bis acrylamide, tris base, sodium hydroxide (NaOH), hydrochloric acid (HCL), tween 20, nonfat milk, tamed, aluminum per sulphate, sodium dodecyl sulphate (SDS), pyrogallol, Ellmen's reagent, acetic acid, phosphate buffer saline (PBS), Bradford reagent, bovine serum albumin (BSA), were purchased from Sigma Aldrich. LBDE from Korea. During experiment deionized water was used. A digital pH meter model 510 (Oakton, Eutech) equipped with a glass working electrode and a reference Ag/AgCl electrode, Western blot apparatus from Bio-Rad, UV–visible spectra were recorded with a UV-2800 BMS Scientific Technical Corporation (PVT), Ltd, Sonicator, Centrifuge and digital balance were used.

3.2 Free Radical Scavenging Activity:

Free Radical Scavenging activity of Lirioresinol B Dimethyl Ether was done to measure its antioxidant activity by using DPPH assay. 1, 1-Diphenyl-2-picryl-hydrazyl (DPPH) possessing free radical is used to calculate free radical scavenging activity of a compound with which it reacts. This assay was performed by making a stock solution of DPPH of 10mM. This solution was purple colored and due to light sensitive nature of DPPH, it was kept in dark. While Lirioresinol B Dimethyl Ether was dissolved in Dimethyl Sulfoxide (DMSO) to make 1mM solution. To compare anti-oxidant activity of LBDE, four different concentrations of solutions were prepared. Composition of each reaction is given in table 3.1

Table 3.1 Composition of DPPH reaction

Reactions	DPPH sol	LBDE Conc	Water
1	100 ul	50 ul	1.35 ml
2	100 ul	100 ul	1.3 ml
3	100 ul	150 ul	1.25 ml
4	100 ul	200 ul	1.2 ml

For each reaction, after adding above mentioned quantities of DPPH, LBDE and water, it was kept in a dark place. Reaction took place after 30 to 35 minutes, with change in color from dark purple to light yellow. This reaction was observed under UV-VIS spectrophotometer (UV-2800) at 517 nm. For preparation of control reaction, DPPH solution (100µl) and water (1.4ml) were mixed, while blank reaction contained 200 µl of LBDE and 1.3ml of water as a solvent.
Absorbance values at the time of reaction were noted and used to calculate percentage antioxidant activity (%AA) by formula given below:

$$\%AA = \frac{100 - [(Abs\ of\ sample) - (Abs\ of\ blank)] \times 100}{Abs\ of\ control}$$

3.3 Animals

26 healthy, 2-3 weeks old BALB/C male mice were purchased from the animal facility of National Institute of Health (NIH) Islamabad, Pakistan. All mice were given the same environmental conditions, with proper ventilation, moisture, and a day and night cycle of 12 hours respectively was maintained in the animal facility of department of Biomedical Engineering and Sciences, School of Mechanical and Manufacturing Engineering, National University of Science and Technology. They were given free access to food and water. A Before the experimentation procedures all mice were given one-week acclimatization period.

3.4 Experimental design

Mice were categorized into 4 groups: five mice of control group labelled as group 1, while three experimental groups of seven animal per group were labelled as group 2 (DEN+CCl-4), group 3

(DEN+CCl4+LBDE), and group 4 (DEN+CCl4+5-FU). 5-FU was used as the positive control as it is already in use for treatment of liver Fibrosis.

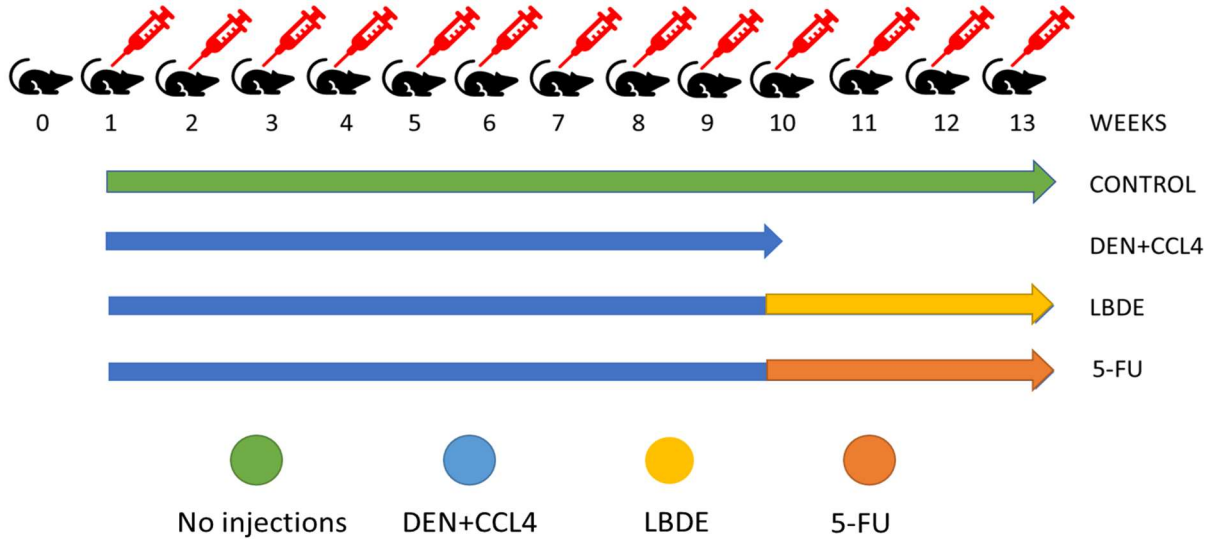

Figure 3.1 Graphical representation of Experimental design

3.4.1 Weight analysis

Mice weight was monitored once every week till the end of the experiment.

3.4.2 Induction of fibrosis

For the induction of hepatic fibrosis except group 1 which was designated as control all three groups were intraperitoneally administered with 10 % CCl4 diluted in Olive Oil at a dose of 0.2ml/kg and 1% DEN diluted in normal saline at a dose of 30mg/kg on alternate days of week for a period of ten consecutive weeks.

3.4.3 Treatment

After 10 weeks of treatment with DEN+CCl4 group 3 mice were given Intra venous injections of LBDE at a dose of 50mg/ kg and group 4 mice were intraperitoneally administered with 5-FU at a dose of 30mg/kg for a period of 3 weeks respectively.

Figure 3.2 Administration of intra peritoneal injection to the mice.

3.4.4 Euthanization

Mice in group 2 after 24 hrs. of last injection of DEN and mice in Group 3, and 4 after 24 hrs. of last injection of LBDE and 5-FU respectively were euthanized. The control group mice were also euthanized after 13th week of the experimental protocol.

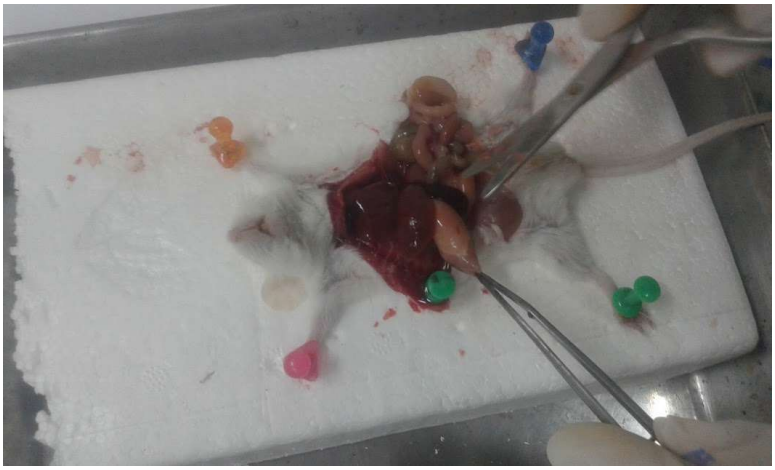

Figure 3.3 Dissection of mice

3.4.5 Sample storage

Blood was collected from the cardiac puncture in serum separation tube, after 5-times gentle shaking tubes were place to rest for a while and then they were centrifuged at 3000 rpm for 5 min. serum was separated in this way and was collected in 1.5 ml E-tubes and stored at -4 degree centigrade. Right lobe of liver from each group was placed in 70% formalin and stored at room

temperature for histopathology while the rest of the liver tissue was stored at -80 degree centigrade in the cryo-freeze for biochemical analysis and protein quantification.

3.5 Hepatotoxicity

For the quantification of liver injury biomarkers i-e Alkaline Phosphatase (ALP), Alanine Aminotransferase (ALT) and total bilirubin, serum stored in E tube were sent to the diagnostics labs at Atta-Ur-Rahman School of Applied Biosciences

3.6 Histopathology

Right lobe of liver tissues obtained from mice that were kept in 10% formalin solution remained stored at room temperature until they were sent to the Islamabad diagnostic center for liver biopsy. Prepared slides of H&E stained liver tissue were then obtained and were viewed under microscope at 10X and 40X resolution for observation of liver cell morphology. The services of Histopathology lab, Islamabad Diagnostic Centre were used.

3.7 Protein Isolation

Liver tissue samples stored in Cryo-freezer were used to isolate protein for Biochemical Assays, Protein Quantification and Western blot analysis. 0.2g of liver tissue was taken out in falcon tube containing 1ml of Lysis buffer to cause cell lysis for extraction of proteins. Tissues was homogenized by rigorous pipetting of 40 to 50 minutes, afterwards this homogenate was subjected to sonication using probe sonicator, and the facility of probe sonicator was obtained by Chemistry lab of School of Natural Sciences, NUST (throughout the whole homogenization steps samples were placed on ice tray). When tissue was finely homogenized in the form of solution, it was then centrifuged. Centrifugation was carried out at 10,000 rpm for 15 minutes at -4 degree centigrade. After centrifugation supernatant was collected while pallet at the bottom of tube was discarded. This step was repeated thrice to avoid any cellular contents other than proteins. The final solution of extracted proteins was then stored in Cryo-freezer for further use.

3.8 Oxidative stress

For the determination of oxidative stress in the fibrotic liver tissue and check how LBDE has acted against this stress a series of biochemical assays were done namely:

- Thio-Barbituric Acid Reactive Substances Assay (TBARS) (Anti lipid per oxidation)
- Superoxide radical scavenging assay (SOD)
- Glutathione assay (reduced glutathione)
- Bradford assay (protein detection)
- Western blot assay

3.8.1 Thio-Barbituric Acid Reactive Acid Substance Assay

For the determination of degree of lipid peroxidation in the mice liver tissue, thiobarbituric acid reactive substance assay was used. The principle of this method is the formation of malondialdehyde as a product of peroxidation of lipid. This MDA in the assay reacts with the thiobarbituric acid (TBA), because of this reaction a pink chromogen the thiobarbituric acid reactive acid substance is produced.

E tubes were labelled, and the reaction mixture for this assay i-e TBA- TCA (trichloroacetic acid) were added, to which tissue homogenate was added, and the tubes were heated at 60 degree centigrade for a period of 15 minutes. Samples were prepared in a way that 3 samples were made from each experimental group. The tubes were centrifuged (1000 rpm for 10 mins) pallet was discarded and the supernatant was collected. The concentration of MDA was measured at 532nm under UV-VIS spectrophotometer, blank solution did not contain sample.

The following formula was used for its calculations:

$$\textit{Concentration of MDA} = \frac{\textit{Absorbance at 532}}{\textit{1.56*10\^5}}$$

Figure 3.4 TBARS assay

3.8.2 Reduced glutathione Assay

When oxidative stress is increased in the cell the antioxidant enzymes concentration also increases in the cell, to cop up with this stress, reduced glutathione is one of them. The principle of this assay is that the reduced form of glutathione contains free thiol groups Ellman's reagent which is chemically a 5,5'-dithiobis-2-nitrobenzoic acid reacts with this free thiol group and produce yellow color. The production of more chromogen is in direct proportion to the amount of reduced glutathione.

Falcon tubes were labelled and 5% trichloroacetic acid (TCA) dissolved in DMSO was added into it, liver tissue homogenate was then added. Samples were prepared in a way that 3 samples were made from each experimental group. Centrifugation was done at 3000 rpm for 15 min to supernatant, Ellman's reagent in 1% acetic acid, and 3ml phosphate buffer saline (PBS) were added, blank solution did not contain sample. Absorbance for each sample was observed under UV-VIS at 412 nm.

The following formula was used for its calculations:

$$Concentration = \frac{Absorbance\ at\ 412\ *Dilution\ Factor}{Extinction\ coefficient\ (13,600/M/cm)}$$

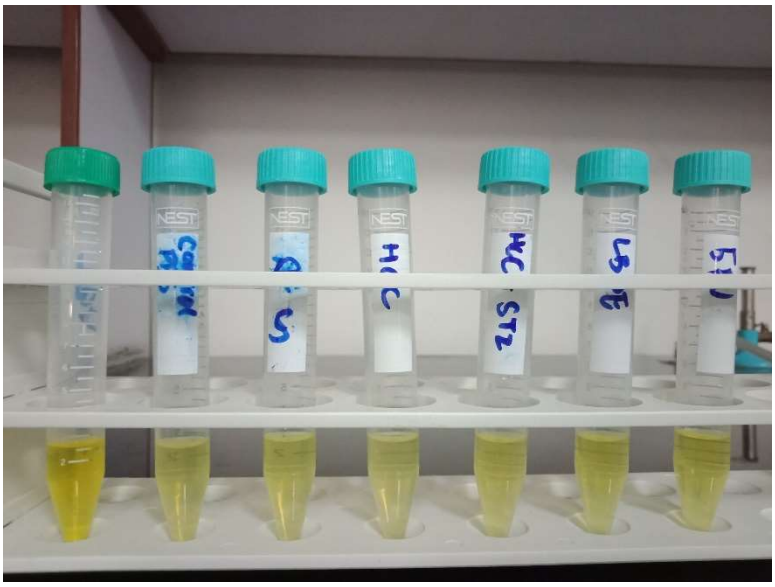

Figure 3.5 Glutathione Assay

3.8.3 Super-Oxide Dismutase Assay

Super oxides dismutase is another group of antioxidant enzymes that converts the radicals of super oxide into H_2O_2 (hydrogen peroxide) and O_2 this hydrogen peroxide in turn further neutralized by the action of catalases and peroxidases. The principle is that SOD prevents the Auto-Oxidation of Pyrogallol.

Falcon tubes were labelled, TRIS-EDTA buffer pH 8 was added, to which 20 mM pyrogallol and tissue homogenate of liver was added. Samples were prepared in a way that 3 samples were made from each experimental group. To the blank solution instead of sample double distilled was added. Absorbance for each sample was observed under UV-VIS at 380nm. The experiment was repeated thrice. The following formula was used for its calculations:

$$\% \text{ Inhibition of pyrogallol} = \frac{\text{change in OD control - change in OD test} * 100}{\text{Change OD Test}}$$

$$\text{SOD activity (U/ml)} = \frac{\% \text{ inhibition of pyrogallol}}{50}$$

Figure 3.6 Superoxide dismutase assay

3.9 Bradford Assay

Assay for protein quantification using Bradford reagent is called Bradford protein assay. To prepare a standard curve, Bovine Serum Albumin (BSA) is used. Six different concentrations of BSA and water were added in 200 µl of Bradford reagent to make final volume of 1ml.

Table 3.2 Values for BSA standard

µg/ml	Bradford Reagent (µl)	BSA (µl)	H2O (µl)
0	200	0	800
2	200	20	780
4	200	40	760
6	200	60	740
8	200	80	720
10	200	100	700

These reaction mixtures were then vortexed and incubated at room temperature for 5 to 10 minutes. One by one, each reaction mixture was checked for its absorbance under UV-VIS spectrophotometer at 595nm. These absorbance values were then plotted to form a BSA standard curve

3.10 Protein Quantification

Protein quantification assay was done by preparing reaction mixtures of samples, Bradford reagent and water. Samples were prepared in a way that 3 samples were made from each experimental group. Each sample contained 200µl of Bradford reagent, and 798 µl of water. All the sample mixtures prepared in E-tubes were labelled accordingly and then vortexed and incubated at room temperature for 5 to 10 minutes. Absorbance for each sample was observed under UV-VIS spectrophotometer at 595nm.

3.11 Western blot Analysis

3.11.1 Loading sample

Table 3.3 Loading sample values for western blot

Groups	**abs**	**2ug**	**1ug**	**50**	**H20**	**4X**	**Total**
Control	0.41	12.5862069	6.2931034	7.9452054	2.0547	3.3	13.3
DEN+CCl4	0.47	14.4827586	7.241379	6.9047619	3.0952	3.3	13.3
LBDE	0.36	11.0057471	5.5028735	9.0861618	0.9138	3.3	13.3
5-FU	0.35	10.6896552	5.3448275	9.3548387	0.6451	3.3	13.3

Loading sample values as given in table 3.2 were estimated using the equation obtained from regression line of standard curve. Sample was prepared in a way that to each sample 4X buffer and water was added, the tubes were then vortexed and exposed to high temperature i-e 95 ^{o}C for 5 min. The samples tubes were centrifuged after heating and now were ready to be loaded.

3.11.2 SDS Polyacrylamide Gel Preparation:

Optimization of SDS gel preparation led to use of 10% gel protocol. Assembly of plates was done before the preparation of gels. For gel electrophoresis two different types of gels are used, the first is the separating gel which separated the proteins and the second is the stacking gel which is poured above the separating gel and sample loading wells are formed in it.

3.11.3 SDS PAGE Electrophoresis:

The plates after the formation of gels were assembled and placed in the electrophoresis tub, 1X running buffer was added. The prepared samples, low range protein marker and loading buffer were then loaded in the wells. Gel was run in the for 130 minutes at 90V.

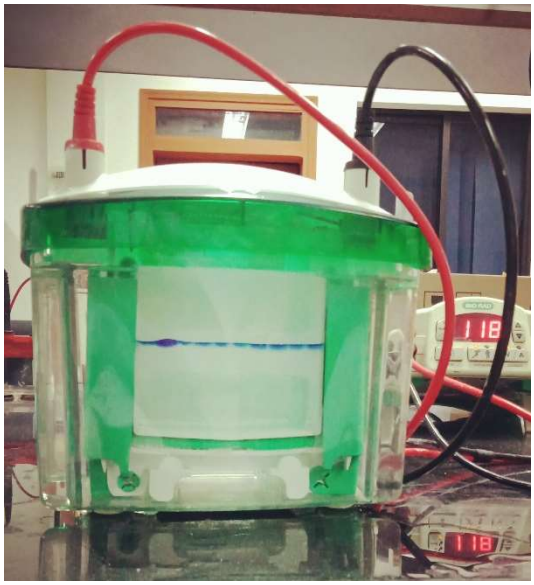

Figure 3.7 Gel running in gel electrophoresis

3.11.4 Gel Transfer:

After completion of gel running, protein bands were transferred to Nitrocellulose membrane (NC). NC membrane and Gel were placed together in a way that sponges and blotting paper were on both sides like a sandwich. This sandwich was placed in transferring apparatus, connected to positive and negative terminals in the presence of 1X transfer buffer. Transferring from Gel to NC membrane took 25 minutes for each gel, while voltage was kept 90V.

3.11.5 Band detection

As soon as transfer was completed, NC membrane was dipped in Ponceau solution to visualize protein bands. This staining was done to find whether protein bands on gel have transferred properly to NC membrane or not. After visualizing bands, NC membrane was marked for required protein detection according to protein marker bands.

3.11.6 Blocking

Non-fat milk solution was prepared by mixing 2g non-fat milk in 40ml PBS solution. After staining with Ponceau solution, NC membrane was washed with distilled water and then dipped in 5 % non-fat milk and shacked for 1 to 2 hours at 4ºC. This step was done to avoid non-specific binding of proteins.

3.11.7 Primary antibody

Santa-Cruz and Cell Signaling anti-bodies were used for protein binding and detection in this study, Primary anti-bodies included NF-kB, and COX-2. Primary anti-bodies were diluted with 1X TBS solution in 1:1000.

3.11.8 Treatment with Primary Anti-bodies:

After blocking step, NC membrane was treated with primary anti-body. This was done by gently shaking of NC membranes dipped in prepared primary anti-body solution overnight at 4ºC.

3.11.9 Washing with TBST:

After the completion of incubation period with the primary antibodies, NC membrane was washed with TBST solution for 5 times. Washing was done for 5, 5, 10, 10 and 10 minutes consecutively to remove unbound primary anti-body to avoid false detection.

3.11.10 Secondary Anti-bodies:

Secondary anti-bodies were diluted in 1X TBS solution in 1:2000. Secondary anti-bodies were of rabbit, and goat and the incubation period were from 3-4 hrs. Use of secondary anti-body was made according to the source of primary anti-body. While incubation time, gentle but continuous shaking was insured by commercially designed shaker.

3.11.11 Washing with TBST

Again, after completion of the incubation period with the secondary antibodies, NC membrane was washed with TBST solution for 5 times. Washing was done for 5, 5, 10, 10 and 10 minutes consecutively to remove unbound secondary anti-body. Secondary antibodies bound to the primary antibodies.

3.11.12 Enhanced Chemiluminescence

For visualization the Enhanced Chemiluminescence-Kit (ECL-kit) was used, this kit consisted of two different solutions which were mixed in a 1:1 concentration. The NC membrane strips were dried by keeping them on a tissue paper and were then placed on the X-ray cassette. After neatly placing the strips on the cassette the prepared ECL solution was poured onto it and was left for 2-3 mins at room temperature so that the solution be properly absorbed by the NC membrane.

3.11.13 X-Ray Films

The excess of the ECL solution anywhere else on the cassette except the NC strip was also dried using tissue paper and the strips were covered with a transparent plastic sheet any wrinckle or bubble in the sheet was also removed. Afterwards in the dark room X-films were placed carefully on the strips, 2 X-Ray films were placed on top of each other. The X-Ray Cassette was closed and was left there for 45min-1 hr.

3.11.14 X-Ray Development

To Develop X-Ray films the commercially available developer solution was used in a ratio of 1:9 and to fix the X-Ray films the commercially available fixer solution in a ratio of 1:9 was used. After 1 hour in the X-Ray cassette the cassette was opened in the dark room and the first X-Ray film was taken out. The film was dipped into the developer solution for few seconds and then was washed by dipping into the tray containing water again after few seconds it was dipped into the fixer solution and then the film was let to dry. The second film of X-Ray was kept in the cassette and was taken out after 24 hrs.

3.11.15 Visualization

For the visualization, after drying step the films were viewed for visible bands.

4 Results

4.1 Body weight analysis

Body weight of mice was analyzed during the whole course of this study. Figure 4.1 shows that the initial weight of mice was almost same at time of start of the study, as hepatic fibrosis was induced by the induction of DEN and CCL4 there was a gradual decrease in the body weight in all experimental group except the control. Furthermore, the body weight start increase sing after the induction of fibrosis. The body weight kept on increasing after the administration of LBDE and 5-FU.

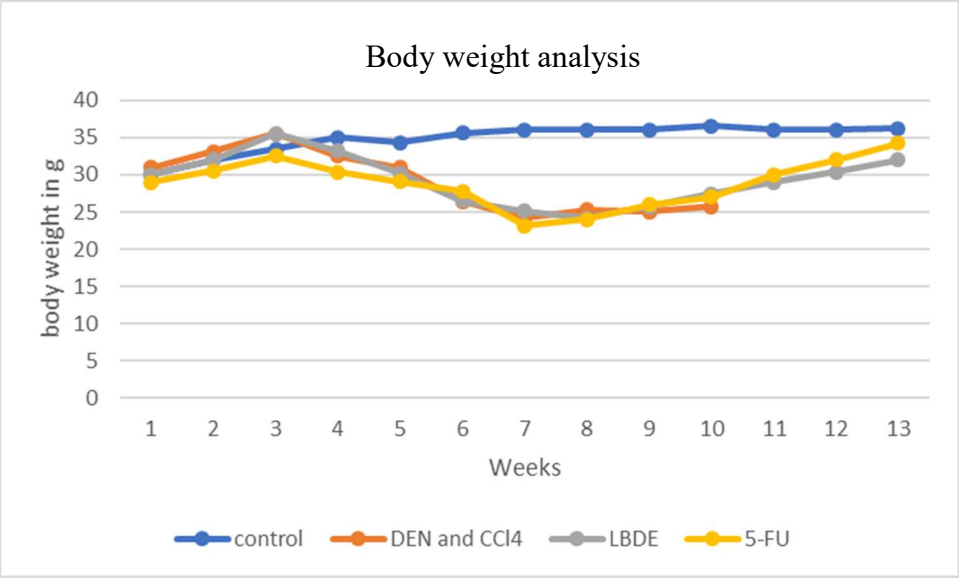

Figure 4.1 Body weight analysis chart

4.2 DPPH ASSAY

Lirioresinol B Dimethyl Ether was evaluated for its anti-oxidant activity by DPPH assay. Four different concentrations of Lirioresinol B Dimethyl Ether were compared for anti-oxidant activity. Three readings were obtained against each concentration and results showed that antioxidant activity was increasing with increase in concentration, as shown in figure 4.2. This figure shows that percentage anti-oxidant activity (%AA) at 50µM was 73%, at 100 µM was 79.79%, 1t 150µM was 90.1% and at 200µM was 99%.

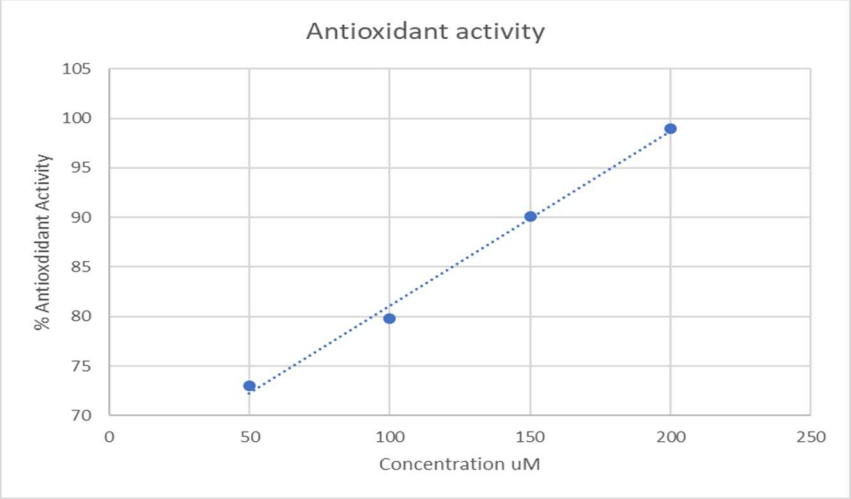

Figure 4.2 Percentage anti-oxidant activity of LBDE at different concentration

4.3 Liver function tests

Serum was used for the analysis of liver function biomarkers, three biomarkers were analyzed i-e Alanine Aminotransferase (ALT), Alkaline Phosphatase (ALP), and total bilirubin.

4.3.1 Serum Alanine Transaminase (ALT)

Results showed that, level of ALT was increased to three-folds when DEN and CLL4 were administered as shown by group 2 in figure 4.3.

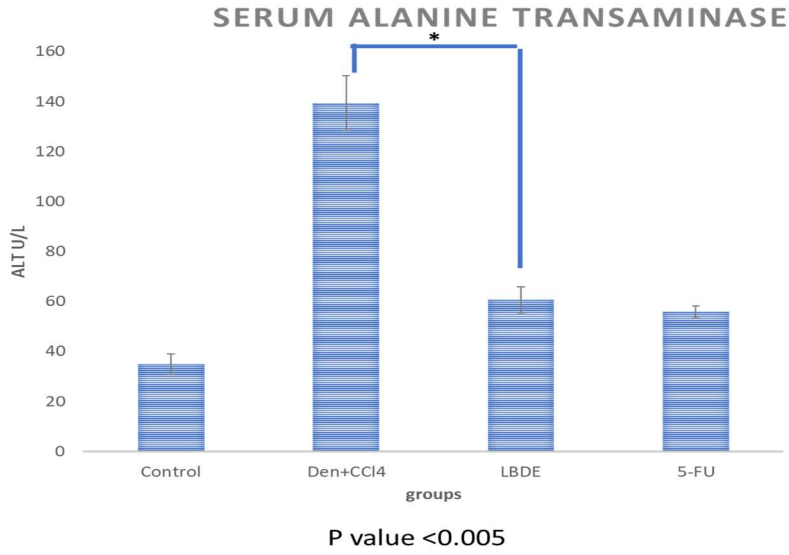

Figure 4.3 Serum Alanine Transaminase level.

Figure 4.3 shows serum level of alanine transaminase with the camparassion of group 2 (DEN+CCL4) and group 3 (LBDE). The level of ALT was reduced back near to normal range when treated with LBDE and same trend was observed in the control positive group (Group 4). For statistical analysis, One Way ANOVA was performed and comparison among experimental groups was significant, $P < 0.005$.

4.3.2 Serum Alkaline Phosphatase

Results showed that, level of ALP was increased when DEN and CLL4 were administered as shown by group 2 in figure 4.4.

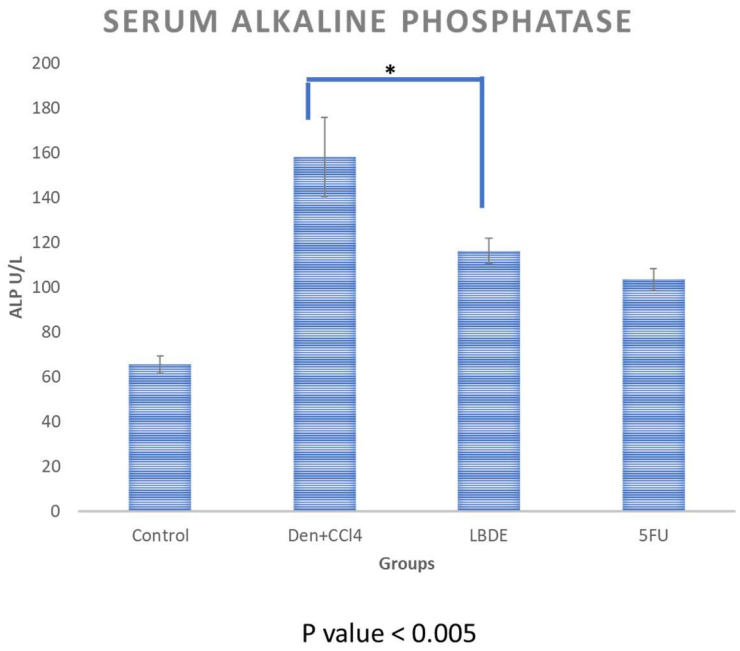

Figure 4.4 Serum Alkaline phosphatase level.

Figure 4.4 shows the level of serum alkaline phosphatase with the camparassion of group 2 (DEN+CCL4) and group 3 (LBDE). The level of ALP was reduced back near to normal range when treated with LBDE and same trend was observed in the control positive group (Group 4). For statistical analysis, One Way ANOVA was performed and comparison among experimental groups was significant, $P < 0.005$.

4.3.3 Total Bilirubin

Results showed that, the level of total bilirubin was increased three-folds in the group 2 which was the DEN+CCL4 group and after treatment with LBDE and 5-FU in group 3 and 4

respectively the level to total bilirubin was reduced as shown in the figure 4.5 were administered as shown by group 2 in figure 4.5. For statistical analysis, One Way ANOVA was performed and comparison among experimental groups was significant, $P < 0.005$.

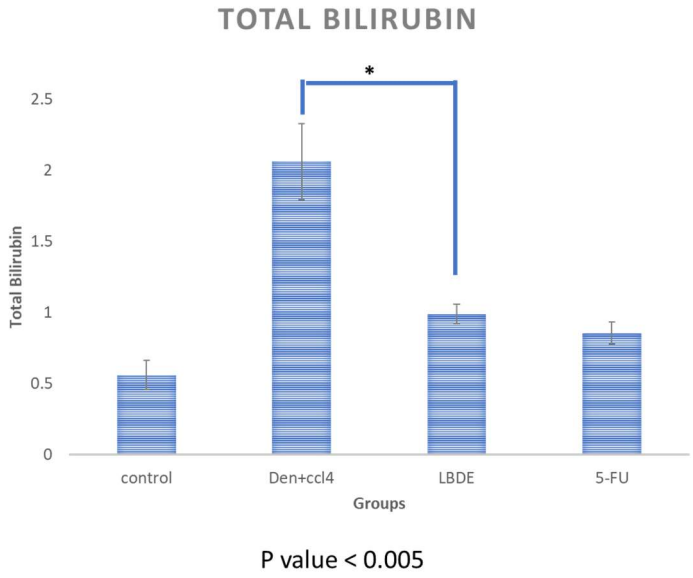

Figure 4.5 Serum level of Total bilirubin.

Figure 4.5 shows the serum level of total bilirubin with the camparassion of group 2 (DEN+CCL4) and group 3 (LBDE).

4.4 Histopathological Results

For liver histopathology prepared slides obtained from Islamabad diagnostic center were studied under the microscope and images were taken using Pixel pro software. As shown in the figure 4.6 the liver cells of control group had normal architecture and with no marked inflammatory signs normal morphology of hepatocytes is evident from the image ‘a’. The group 2 which is DEN+CCL4 treated group had severe portal vein inflammation with distorted morphology of hepatocytes and necrotic cells all visible in the slides confirm the induction of hepatic fibrosis in this group. Group 3 which is the LBDE treated group shows healing hepatocytes and the effects of severe inflammation are reversed in this group as shown in the image ‘c’ with no signs of inflammation and dysplasia. Also, the control positive group that is the group 5-FU treated group shows the healing hepatocytes and less to no signs of inflammation.

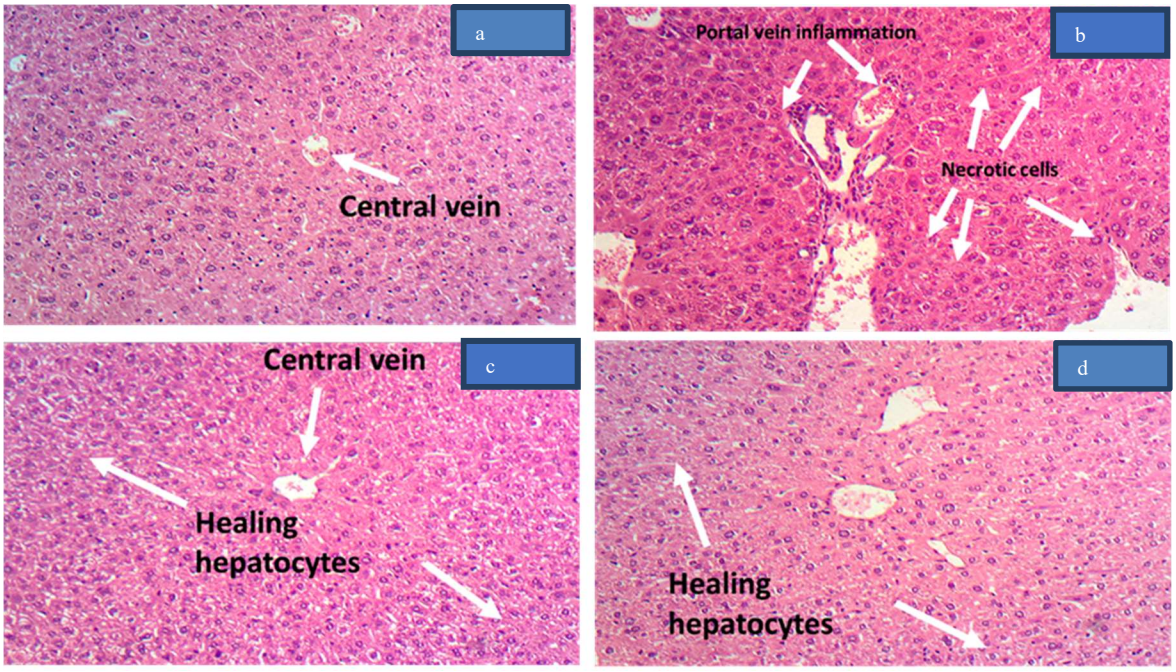

Figure 4.6 The histopathology of live tissue of all four experimental groups.

Figure 'a' shows the histology of group 1 (control group) mouse which shows the normal morphology of the liver, with the normal central vein. 'b' shows the histology of group 2 (DEN+CCL4 treated) mouse which shoes severe portal vein inflammation and necrosis of the hepatocytes. 'c' shows the histology of group 3 (LBDE treated group) mouse with the inflammation almost over and the surrounding healing hepatocytes. 'd' shows the histology of group 4 (5-FU treated group) mouse the surrounding healing hepatocytes. All slides were observed under 10X magnification.

4.5 TBARS

As shown in the figure 4.7, MDA concentration in the hepatic fibrosis group that is DEN+CCL4 is significantly higher than the normal group while when the mice were treated with LBDE and 5-FUin the 3rd and the 4th group respectively the MDA concentration is significantly decreased, and it is more towards the normal range. The P value is less than 0.005 in the DEN+CCL4 and LDE treated group evaluated by t-test.

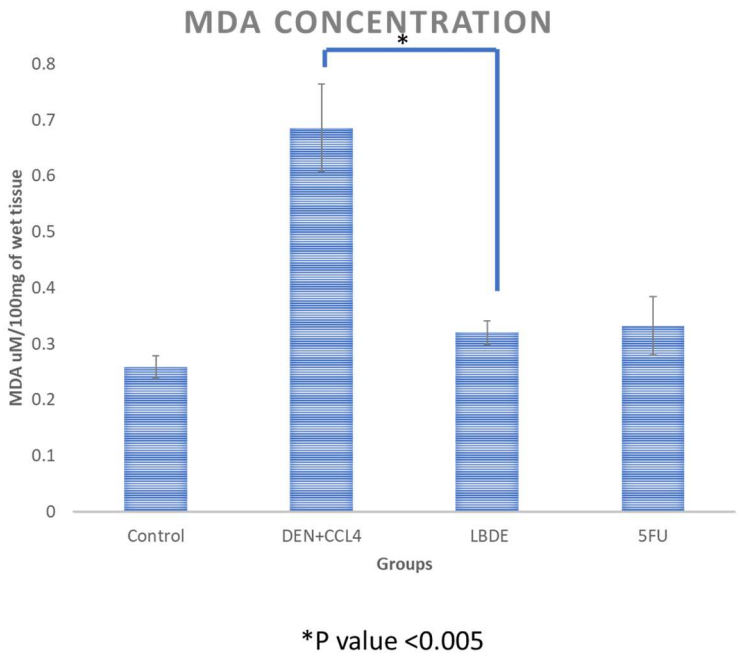

Figure 4.7 Concentration of MD.

Figure 4.7 shows that the control has the normal levels of MDA, while the DEN+CCl4 treated groups has the elevated level of MDA which is evident of severe inflammation and high levels of lipid per-oxidation. LBDE and 5-FU (control positive) has the decrease in the level of MDA.

4.6 SOD

The oxidative stress biomarkers, the antioxidant enzyme Super oxide dismutase levels were tesed by the percent inhibition of pyrogallol, decreased SOD activity in the diseased group showed that the oxidative stress had surpassed the antioxidant enzymes as shown in the figure 4.8. LBDE group had a significantly high level of dismutase's activity as compare to the hepatic fibrosis group, same trend can be seen in the control positive 5-FU. The P value is less than 0.005 in the LBDE and DEN+CCL4 treated group evaluated by t-test.

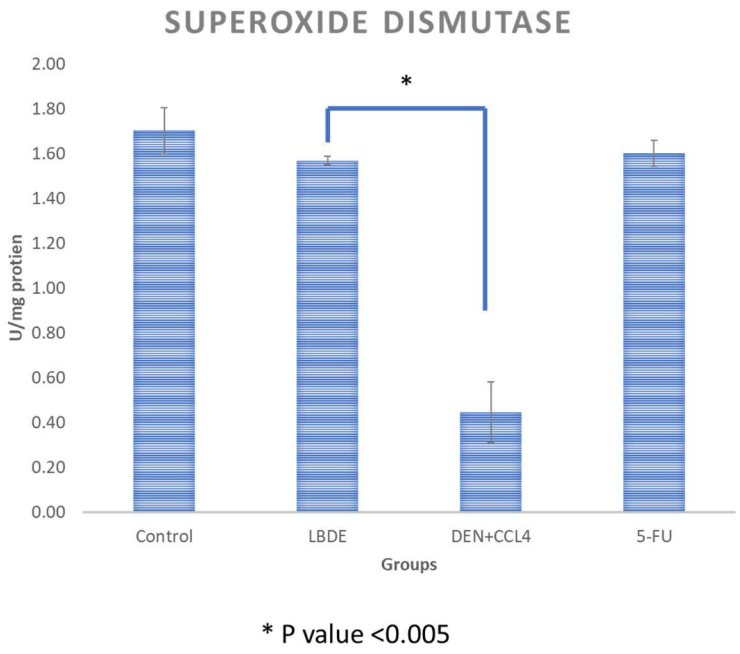

Figure 4.8 Super oxide dismutase concentration.

Figure 4.8 shows that the control has the normal levels of SOD, LBDE treated group has the elevated level of SOD showing the effectiveness of LBDE against severe inflammation, while the DEN+CCl4 group has the decreased level of SOD which is evident of severe inflammation and high levels of oxidative stress.

4.7 Reduced Glutathione (GSH)

The reduced glutathione is an antioxidant enzyme its level was decreased in the diseased group showing that the oxidative stress has surpassed the antioxidant enzymes as shown in the figure 4.9. LBDE group has a significantly high level of reduced glutathione as compare to the hepatic fibrosis group, same trend can be seen in the control positive 5-FU. The P value is less than 0.005 in the LBDE and DEN+CCL4 treated group evaluated by t-test

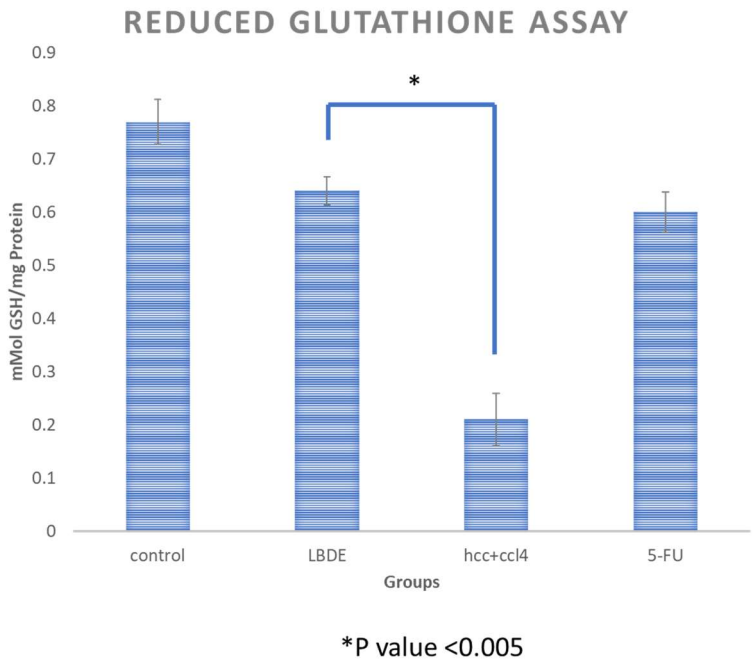

Figure 4.9 Reduced glutathione concentration.

Figure 4.9 shows that the control group has the normal levels of GSH, LBDE has the elevated level of GSH showing the effectiveness of LBDE against severe inflammation, while the DEN+CCl4 has the decreased level of GSH which is evident of severe inflammation and high levels of oxidative stress

4.8 Bradford standard curve

The standard curve of BSA was obtained from this standard curve regression line with the value of R^2 =0.99 was used to quantify protein content in the samples, Figure 4.10.

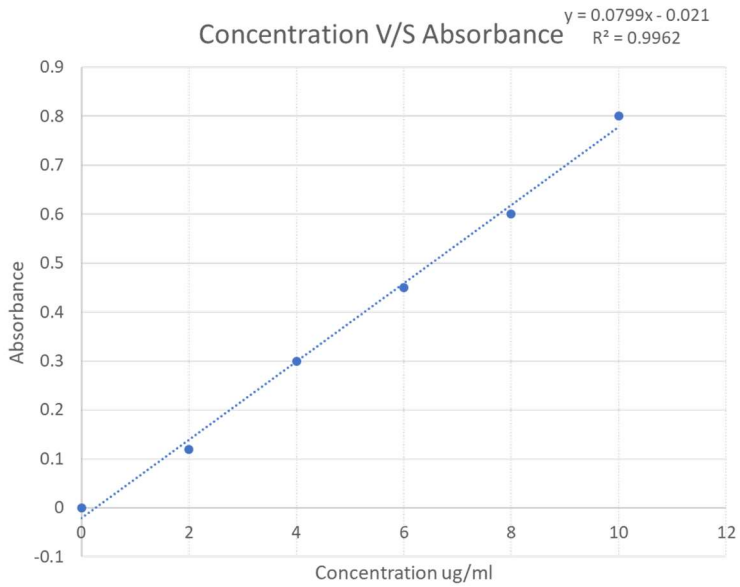

Figure 4.10 BSA standard curve used to quantify protein.

4.9 Western blot assay

Results of Western Blot technique were visualized at two different stages, firstly at stage of staining the NC membrane with Ponceau solution for detection of proper protein transfer from gel to NC membrane and then final detection was done by X-ray analysis.

4.9.1 NC Membrane staining

When the NC membrane was stained with Ponceau solution the protein bands were visible on the NC membrane

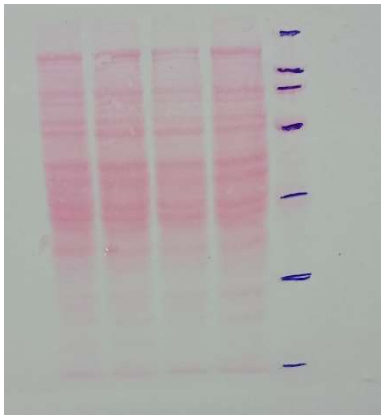

Figure 4.11 Ponceau stained NC membrane

4.9.2 Final Protein detection

The final expression of protein was determined by performing the X-ray analysis of the NC membrane.

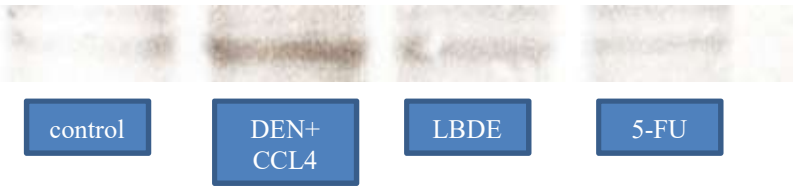

Figure 4.12 Expression of NF-$_k$B.

Figure 4.12 shows that the expression of NF-$_k$B, in DEN+CCL4 group the expression of NF-$_k$B is more and in LBDE and 5-FU treated experimental groups the expression of NF-$_k$B is low.

Figure 4.13 Expression of COX-2.

Figure 4.13 shows the expression of COX-2, in DEN+CCL4 group there is a high expression of cox-2 but as compare to that LBDE and 5-FU has a low expression of COX-2.

5 DISCUSSION

In a variety of hepatic diseases the hepatic fibrosis is present, the link between constant fibrosis and the development of hepatic cancer has been well established (Nissen & Martin, n.d.) Intruding the process of hepatic fibrosis or even reversing hepatic fibrosis is a new approach for the progression of hepatic fibrosis to the stage of hepatocellular carcinoma. As far as the process of reversing hepatic fibrosis is concern it is still under research and not well understood.

In this in-vivo study of Lirioresinol B Dimethyl Ether (LBDE) which is a bioactive chemical compound, obtained from seed oil of Magnolia Fargesii, Family of Magnolieceae was conducted for its hepatoprotective effect on hepatic fibrosis in Balb/c mice. DEN and CCl4 were used as hepatotoxins in this study and after the induction of hepatic fibrosis LBDE was administered, as the anti-inflammatory role of LBDE is reported against bone destruction, it also inhibits chronic inflammation by downregulating Receptor activator of NF-κB Ligand (RANKL), to stop inflammatory vicious cycle (Jun et al., 2014).

The beneficial effects of LBDE in the treatment of hepatic fibrosis induced chemically were quite evident from the changes in the liver morphology. Changes in liver morphology were an obvious of the liver fibrosis in the diseased group but there was a generalized improvement in the morphology of liver after treatment with LBDE. The preventive outcomes of LBDE may be activated by stopping the hepatic stellate cell activation.

The role of oxidative stress in the development of hepatic fibrosis is evident from the work of (Heindryckx, Colle, & Van Vlierberghe, 2009; Liu et al., 2006; VIDELA et al., 2004; White, Kanwal, Jiao, & El-Serag, 2016), by the modulation of various signaling pathways. Malondialdehyde reacts with the thiobarbituric acid (TBA) and its concentration determine the oxidative stress (Noeman, Hamooda, & Baalash, 2011) as compare with the DEN+CCL4 group there was a considerable decrease in the concentration of MDA in the LBDE treated group showing the decrease in the oxidative stress. Super oxides dismutase and reduced form of glutathione both belongs to the antioxidants group of enzymes (Noeman et al., 2011) analysis of both GSH and SOD showed the effectiveness of LBDE against severe inflammation, the DEN+CCl4 had the decreased level of GSH and SOD which is evident of severe inflammation and high levels of oxidative stress.

In this study LBDE successfully prevented hepatic fibrosis by suppressing the NF kappa B and Cox-2 pathway. Lirioresinol B Dimethyl Ether was found to downregulate key inflammatory protein NF-κB and Cox-2 in experimental group treated with Lirioresinol B dimethyl ether after DEN+CCL4. NF-κB is responsible for transcription of anti-apoptotic proteins and inflammation causing genes e.g., COX-2. By Inhibition of NF-κB expression, its translocation into nucleus and transcription of genes responsible for inflammation, cell survival, cell proliferation and metastasis was inhibited (Greten et al., 2004).

In NF-κB pathway, I-κB being inhibitory protein plays a critical role in inhibition of NF-κB regulated inflammatory vicious cycle. I-κB inhibits NF-κB from nuclear translocation and transcription of inflammatory genes. But in case of liver injury or inflammation, I-κB is phosphorylated and degraded by proteasome, thus resealing NF-κB for nuclear translocation and anti-apoptotic and inflammatory gene transcription. NF-κB activation is dependent upon phosphorylation of I-κB followed by second step in which I-κB is ubiquitinated and degraded by proteasome. After phosphorylation of I-κB, NF-κB is released in a way that it can freely translocate into nucleus and being transcription factor, activate transcription of inflammation promoting genes e.g., TNF-alpha, COX-2, MMPs etc (Yamamoto & Gaynor, 2004b). As mentioned above, when chronic inflammation establishes, a vicious cycle is started. Inflammatory genes activated by NF-κB further enhance inflammation and continue to activate enzymes responsible for I-κB phosphorylation and degradation followed by NF-κB nuclear translocation (Barnes & Karin, 1997). In this study, Lirioresinol B Dimethyl ether also inhibited phosphorylation and degradation of I-κB, thus restoring its expression levels to inhibit NF-κB activation and inflammation promotion.

The present study reveals that Lirioresinol B Dimethyl Ether have antioxidant qualities and it inhibits the chronic liver conditions by inhibiting downregulating NF-κB and intern upregulating I-κB expressions.

6 CONCLUSION

From this study it is concluded that NF-κB and Cox-2 pathways can be regulated by Lirioresinol B Dimethyl Ether and in this way hepatic fibrosis, which can lead to hepatocellular carcinoma can be inhibited. LBDE downregulate NF-κB by the activation of I- κB, the inhibiton of nuclear translocation of NF-κB will eventually prevent transcription of antiapoptotic proteins and inflammatory genes such as COX-2.

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