Lirioresinol B Dimethyl Ethers Inhibits CCL4-induced Chronic Inflammation and Cell Dysplasia by Preventing NF-kB Expression

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A thesis submitted in partial fulfillment of the requirements for the degree of

MS Biomedical Sciences

Thesis Supervisor:

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Thesis Supervisor's Signature: _____

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Declaration

I certify that this research work titled "*Lirioresinol B Dimethyl Ethers Inhibits CCL4-induced Chronic Inflammation and Cell Dysplasia by Preventing NF-kB Expression"* is my own work. The work has not been presented elsewhere for assessment. The material that has been used from other sources it has been properly acknowledged / referred.

Signature of Student

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Acknowledgements

I am thankful to my Creator Allah Subhana-Watala to have guided me throughout this work at every step and for every new thought which You setup in my mind to improve it. Indeed I could have done nothing without Your priceless help and guidance. Whosoever helped me throughout the course of my thesis, whether my parents or any other individual was Your will, so indeed none be worthy of praise but You.

I am profusely thankful to my beloved parents who raised me when I was not capable of walking and continued to support me throughout in every department of my life and my siblings for their guidance, support, care and prayers.

I would also like to express special thanks to my research supervisor Dr. Adeeb Shehzad for giving me opportunity, guidance, his kind support and encouragement throughout my thesis and also for the courses which he has taught me. I can safely say that I haven't learned any other subject in such depth than the ones which he has taught.

I would also like to pay special thanks to my Lab fellows and friends especially my senior for support and cooperation. Each time I got stuck in something, he came up with the solution. Without their help I wouldn't have been able to complete my thesis. I appreciate their patience and guidance throughout the whole thesis.

I would also like to thank Dr Nabeel Anwar, Dr. Murtaza Najabat Ali, Dr.Umer Ansari and for being on my thesis guidance and evaluation committee and express my special thanks to Dr. Nabeel Anwar for his help. I am also thankful to Dr. Jawad and everyone at Signal Transduction lab, Quaid-e-Azam University, especially Ayesha Awan for their support and cooperation.

Finally, I would like to express my gratitude to all the individuals who have rendered valuable assistance to complete this work.

Dedicated to my exceptional parents and adored siblings whose tremendous support and cooperation led me to this wonderful accomplishment.

Abstract

Inflammation is considered one of the key components in the initiation and progression of Hepatocellular carcinoma (HCC). Upon failure to eliminate Inflammation causing factors which induce acute inflammation, leads to cause chronic inflammation, cell dysplasia, and ultimately cancer. To prevent liver cancer, inflammatory mediators were targeted for inhibition of chronic liver inflammation and liver cell dysplasia in this study. Lirioresinol B Dimethyl ether is an anti-inflammatory phytochemical that was used to modulate expression levels of NF-kB and I-kB. NF-kB is a transcription factor responsible for transcription of inflammatory genes and anti-apoptotic proteins while I-kB is inhibitory protein that inhibits activation and nuclear translocation of NF-kB. Western blot results of this study show that Lirioresinol B Dimethyl Ether down-regulated NF-kB and up-regulated expression levels of I-kB in hepatocytes of experimental group treated with Lirioresinol B Dimethyl Ether. ALT and ALP levels and histopathology results also present that Lirioresinol B Dimethyl ether significantly inhibits chronic liver inflammation and liver cell Dysplasia and prevents cancer development by regulating NF-kB/I-kB activation.

Key Words: *Chronic Inflammation, Liver Cell Dysplasia, Hepatocellular Carcinoma, Lirioresinol B Dimethyl Ether.*

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1. INTRODUCTION

1.1 Inflammation:

Inflammation is defined as a biological response to harmful stimuli that attacks living organism. This harmful stimuli may be an environmental irritant, autoimmune attack, a chemical, or a carcinogen. Body defence system, comprising of immune cells, blood vessels and molecular mediators like cytokines accumulate at the site of inflammation to eliminate the causative factor of inflammation.

1.1.1 Chronic Inflammation:

Body's failure to eliminate inflammation causing factors lead to a condition called chronic inflammation. Chronic inflammation is key factor to various diseases. Chronic inflammation once established, starts vicious cycle that continues to stimulate inflammatory cytokines and transcription factors till complete damage of the organ. Evidences strongly support chronic inflammation progression to cancers (Barnes & Karin 1997).

1.2 Chronic Hepatitis:

Liver being a detoxification organ of the body consists of healthy parenchymal hepatocytes with continuous blood flow. It has ability of regeneration as it possess innate immunity effectors like

Kupffer cells (liver macrophages), Natural Killer Cells, proliferation and survival molecules (Tacke et al., 2009).

1.2.1 Chronic Hepatitis to Hepatic Cancer:

Epidemiological studies had shown an association between chronic inflammation and cancer. However, recent studies have revealed molecular interactions responsible for this association. Inflammatory cytokines and transcription factors promoting expression of inflammatory cytokines are found to be responsible for development of acute inflammation to chronic inflammation and ultimately cancer.

1.3 NF-κB as a Key Inflammatory Regulator:

Key inflammatory regulator transcription factor NF-κB is activated by TNFα in liver inflammation. In studies conducted to understand complex role of NF-κB as a transcription factor, genetic transformation and inactivation of I-κB, NF-κB or by carcinogen like DEN, HCC is induced successfully, showing role of NF-κB in liver Inflammation that subsequently develops into HCC (Colotta et al., 2009) (Maeda et al., 2005). Evidences support important role of Prostaglandins pathway regulated by constitutively expressed COX-2 enzyme in chronic hepatitis. COX-2 over-expression has been reported in chronic hepatitis, cirrhosis and human and 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 of the above mentioned inflammatory cytokines are induced by key inflammatory regulator NF-κB. Angiogenic 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 BCL-2 and thus initiates and up regulates cancer progression pathways particularly. (Gastrointestinal and liver cancers) (Greten et al., 2004) (Pikarsky et al., 2004).

1.4 Liver Cell Dysplasia, initial Cancer Stage:

Liver Cell Dysplasia is reported to be precancerous condition. These morphological lesions in liver cells showing enlargement of cytoplasm and nucleus are frequently found in chronic liver hepatitis and in liver carcinoma with cirrhotic liver. Studies revealed that alpha-fetoprotein (HCC protein marker) is found in dysplastic cells, which shows strong association between dysplastic condition and liver carcinoma. Thus, it is believed that liver cell dysplasia is abnormal condition of liver rather than normal routine lesions (Anthony et al., 1973). Development of dysplastic condition in hepatocytes following chronic liver inflammation with all the characteristic features of chronic hepatitis and liver carcinoma is supported to be a pathological connection between two conditions.

Liver cell dysplasia is characterized by hepatocyte enlargement, nuclear pleomorphism, architectural abnormality of cells and prominent nucleoli.

1.5 Lirioresinol B Dimethyl Ether:

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 (Song & Fisher 1999), possessing 6 methoxyl groups, 8 aliphatic protons and 4 aromatic protons (Kakisawa et al., 1972).

1.5.1 Anti-Inflammatory Activity of Lirioresinol B Dimethyl Ether:

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

extracts responsible for inhibiting ovary-ectomy stimulated osteoporosis progression. Breast Cancer is also known to stimulate bone destruction by hormonal imbalance and cancer metastasis. As a result 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).

1.6 Scope of the Study:

This study was conducted to investigate potential role of plant isolated compound Lirioresinol B Dimethyl Ether in cancer prevention and treatment of chronic liver inflammation and liver cell dysplasia. Chronic inflammation is a condition associated with various diseases like cancer. Underlying pathways for chronic hepatitis are known to be associated with progression into liver cell dysplasia, a precancerous stage and ultimately into hepatocellular carcinoma. Anti-inflammatory agents are known to exhibit anti-cancerous effect as well. Thus, anti-inflammatory phytochemical Lirioresinol B Dimethyl ether used to inhibit chronic liver inflammation and liver cell dysplasia to prevent cancerous condition.

In this study, Lirioresinol B Dimethyl Ether was found to inhibit chronic liver inflammation as well as liver cell dysplasia, thus showing its efficacy against cancer initiation and progression.

This phytochemical is studied very less in the past. But it showed potential anti-inflammatory activity by interfering in the pathway of NF-κB. NF-κB is a key regulator inflammatory transcription factor, responsible for transcription of inflammatory genes promoting cell survival, cell proliferation and anti-apoptotic protein production. Suppression of NF-κB promote down-regulation of various inflammatory cytokines ultimately blocking inflammatory pathways. 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 anti-oxidant 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 anti-oxidant activity.

1.7 Objective:

The objective of this study was to evaluate anti-inflammatory and anti-cancer activity of a phytochemical Lirioresinol B Dimethyl Ether. Carcinogen induced chronic liver inflammation and Liver Cell Dysplasia was treated by LBDE, highlighting its potential for prevention and treatment of cancer. A key inflammatory pathway NF-κB / I-κB was studied and found to be regulated by LBDE and confirmed through morphological examination of liver tissues and expression analysis of proteins and were compared among experimental groups.

2. LITERATURE REVIEW

2.1 Lirioresinol B Dimethyl Ether a phytochemical:

Lirioresinol B Dimethyl Ether is a bioactive chemical compound. It is obtained from *Magnolia Fargesii* (Family *Magnolieceae*) buds (Kakisawa et al., 1972). 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 & Fisher 1999). This phytochemical has been found to possess 6 methoxyl groups, 8 aliphatic protons and 4 aromatic protons (Kakisawa et al., 1972). Structural formula is shown in fig 1.

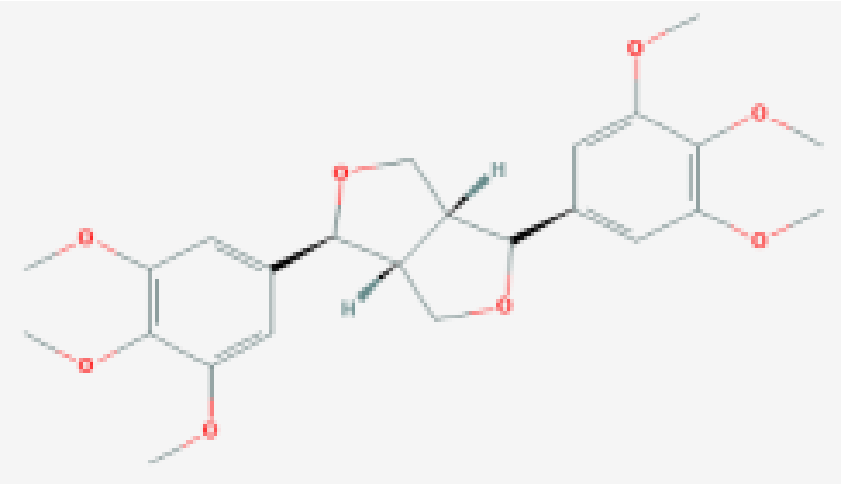

Fig 2.1: Chemical Structure of Lirioresinol B Dimethyl

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).

2.1.1 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. As a result 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).

Phytochemical	Molecular Weight	Molecular Formula	Structural Properties	Solvent
Lirioresinol B Dimethyl Ether	446.496 g/mol	C24H30O8	6 methoxyl groups, 8 aliphatic protons and 4 aromatic protons	Dimethyl Sulfoxide DMSO

Table 2.1: Properties of Lirioresinol B Dimethyl Ether

2.2 Chronic Liver Inflammation:

Inflammation is defined as a biological response to harmful stimuli to our body. It involves immune cells, blood vessels and molecular mediators like cytokines. These cytokines accumulate at the site of inflammation. Failure to eliminate inflammation causing factors lead to chronic inflammation (Miliani et al., 2007). As soon as Chronic Inflammation establishes, a vicious cycle starts and aids in progression of inflammation. Inflammation of liver is known as Hepatitis. Hepatitis can be a result of viral infection or drug toxicity or any other irritant like carcinogen. This condition in selected organs like liver is likely to develop into cancer (Barnes & Karin 1997).

2.2.1: Liver Inflammation and Liver Carcinoma:

Previously, epidemiological studies had shown an association between chronic inflammation and cancer. However, recent studies have revealed molecular interactions responsible for this

association. Inflammatory cytokines and transcription factors promoting expression of inflammatory cytokines are found to be responsible for development of acute inflammation to chronic inflammation and ultimately cancer. Liver being a detoxification organ of the body consists of healthy parenchymal hepatocytes with continuous blood flow. It has ability of regeneration as it possess innate immunity effectors like Kupffer cells (liver macrophages) and Natural Killer Cells as well as cell proliferation and survival molecules (Tacke et al., 2009). Studies have shown that cancers can be prevented by the use anti-inflammatory agents (Mantovani et al., 2008).

2.3 Liver Cell Dysplasia:

Liver Cell Dysplasia is a pre-malignant condition. It is characterized by conditions like Enlargement and unequal sizes of cells, hyper pigmented, abnormal number and sizes of nuclei with prominent nucleoli (nuclear pleomorphism). Cytoplasmic enlargement and lesions in nucleoli that are even visible through low magnification are signs of Dysplastic cells (Anthony et al., 1973).

2.3.1: Dysplasia, Premalignant condition:

Liver Cell Dysplasia is reported to be precancerous condition. These morphological lesions in liver cells showing enlargement of cytoplasm and nucleus are frequently found in chronic liver hepatitis and in liver carcinoma with cirrhotic liver. Studies revealed that alpha-fetoprotein (HCC protein marker) is found in dysplastic cells, which shows strong association between dysplastic

condition and liver carcinoma. Thus, it is believed that liver cell dysplasia is abnormal condition of liver rather than normal routine lesions (Anthony et al., 1973).

Liver Cell Dysplasia is divided into two morphological conditions namely Small Liver Cell Dysplasia (SLCD) and Large Liver Cell Dysplasia (LLCD). In SLCD, there is large cytoplasmic to nuclear size ration, similar to cancerous cells while in LLCD, due to equal increase in size of cytoplasm and nucleus, ration is maintained, that is unlike cancerous cells. However, studies have shown greater association of SLCD with Cancerous cells and revealed LLCD as potential independent risk factor for development of HCC (Libbrecht et al., 2001).

2.4: Molecular Mechanisms:

Evidences show that oncogenes of tumors might stimulate inflammatory cascade by inducing expression of inflammatory cytokines like IL-1β, matrix metalloproteinase (MMPs), Cyclooxygenases (COX-2), pro-apoptotic proteins and angiogenesis promoting proteins (Allavena et al., 2008).

Key inflammatory regulator, a transcription factor NF-κB is activated by TNFα in liver inflammation. 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 Inflammation that subsequently develops into HCC (Maeda et al., 2005).

2.4.1 Role of NF-κB/I-κB in Inflammation:

NF-κB is known to regulate physiology and pathology of liver. Inflammation in 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 (Ghosh et al., 1998). 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 2004), (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 (Barnes & Karin 1997).

2.4.2 Role of NF-κB in Inflammation 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 1994). 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 and 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 of 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 BCL-2 and thus initiates and up regulates cancer progression pathways particularly. (Gastrointestinal and liver cancers) (Greten et al., 2004) (Pikarsky et al., 2004).

3. METHODOLOGY

3.1 Free Radical Scavenging Activity:

Free Radical Scavenging activity of Lirioresinol B Dimethyl Ether was done to measure its anti-oxidant 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 perform 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. In order to compare anti-oxidant activity of LBDE, four different concentrations of solutions were prepared.

Composition of each reaction is given in table 3.1

Reactions	**DPPH soln.**	**LBDE Conc.**	**Water**
1	100µl	50 µl	1.35ml
2	100µl	100 µl	1.3ml
3	100µl	150 µl	1.25ml
4	100µl	200 µl	1.2ml

Table 3.1: Composition of DPPH reactions consisting of four varying concentrations of Lirioresinol B Dimethyl Ether

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 anti-oxidant activity (%AA) by formula given below:

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

3.2 Experimental Models:

3.2.1 Mice:

BALB/C male mice were purchased from University of Peshawar, Pakistan. Total mice were 15 and all were 2-3 weeks old. They were categorized in the form of three groups, 5 mice in each group. Groups were labelled as Control, CCL-4, and CCL4+LBDE. All of them were placed in same environmental conditions, and were given a week to acclimatize. Mice were weighed and weight for each mice was noted. These weights were then averaged to calculate dose of CCL4, Diazald and Lirioresinol B Dimethyl Ether.

3.2.2 Carbon Tetrachloride (CCL4) and Diazald:

Carbon Tetrachloride was used to induce liver Inflammation and Liver Cell Dysplasia in mice.CCL4 was diluted in olive oil by mixing 90ml of olive oil in 10ml of CCL4. Dose of 2-3ml

per week 10 weeks was injected to CCL4 and CCL4+LBDE mice groups. Dose was given on alternate days of week, while control group was left untreated.

Diazald was used to promote effect of CCL4 and accelerate induction of inflammation and progress to cell dysplasia in mice. 200mg/kg per week of Diazald was injected for 3 weeks. Diazald was dissolved in CCL4 (95%) solution and this solution was again diluted as mentioned above.

3.2.3 Treatment with Lirioresinol B Dimethyl Ether:

Lirioresinol B Dimethyl Ether was available in concentration of 1mM. Average weight of mice were used to calculate dose of drug to be injected. As LBDE was injected intraperitoneally, the dose was given according to 50mg/kg of mice.

After induction of liver inflammation and liver cell Dysplasia, Lirioresinol B Dimethyl Ether was injected intraperitoneally for 4 weeks, thrice a week. This treatment was given only to third experimental group labelled as CCL4+LBDE. Treatment injections were administered on alternate days of week.

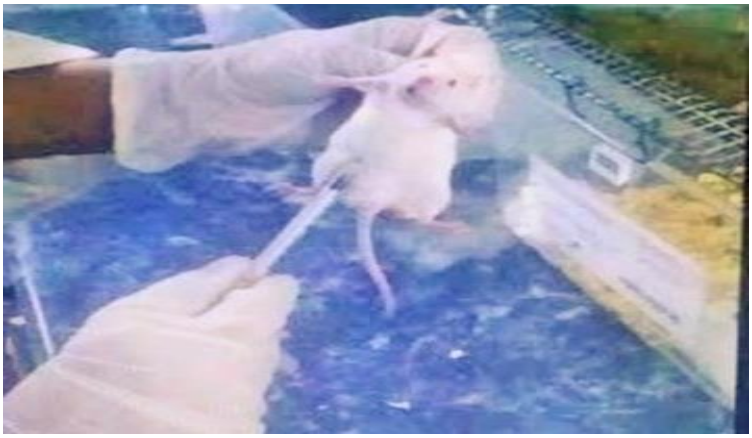

Figure 3.1: Intraperitoneal administration of Lirioresinol B Dimethyl Ether

3.2.4 Mice Sacrificing:

After induction of disease and treatment with Lirioresinol B Dimethyl Ether, mice were sacrificed and blood and liver tissue samples were collected.

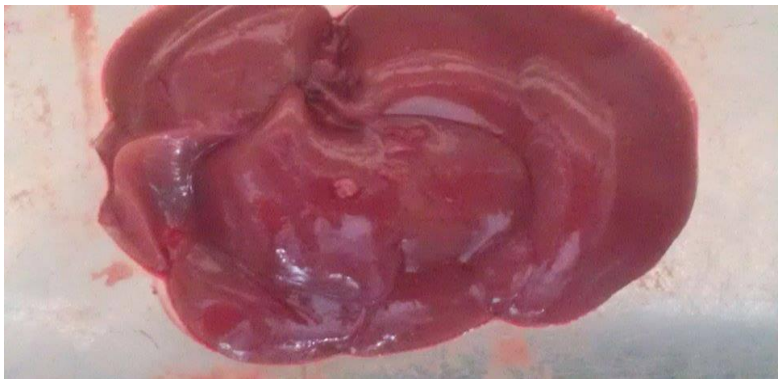

Figure 3.2: Liver of Chronic Inflammation mice model

3.2.5 Sample Storage:

Liver tissue samples that were to be used for protein quantification and protein expression analysis were stored in liquid nitrogen and then transferred to Cryo-freezer at -80ºC, while some liver tissue were stored in formalin for histopathology analysis. Blood samples were stored at -4ºC.

3.3 Liver Function Tests (LFTs):

Liver functioning tests were carried out from blood samples of experimental models (mice). Alkaline Phosphatase (ALP) and Alanine Aminotransferase (ALT) tests were done at Diagnostic lab of Atta-Ur-Rahman School of Applied Biosciences.

3.4 Histopathology:

Liver tissues obtained from experimental mice that were to be used for morphological analysis were sectioned into small fragments and preserved in 10% formalin solution until liver biopsy was performed. Histopathology of liver tissues were carried out by H&E staining and viewed under microscope. 10X and 40X resolution was used for observation of liver cell morphology. Histopathology was conducted at Histopathology lab, Islamabad Diagnostic Centre.

3.5 Protein Isolation:

Liver tissue samples stored in Cryo-freezer were used to isolate protein for protein quantification and western blot analysis.

2µg liver tissue was taken out in falcon tube and 2ml of Lysis buffer was added. Lysis buffer cause cell lysis of tissue for extraction of proteins. Tissues were homogenized by rigorous pipetting of 40 to 50 minutes. 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 and supernatant was collected while pallet at the bottom of tube was discarded. This step was

repeated thrice to avoid cell contents other than proteins. The final solution of extracted proteins was then stored in Cryo-freezer for further use.

3.6 Bradford Assay:

3.6.1 BSA Standard curve:

Assay for protein quantification using Bradford reagent is called Bradford protein assay. In order to prepare a standard curve, Bovine Serum Albumin (BSA) is used. Six different concentrations of BSA and water (shown in table 3.2) are added in 200 µl of Bradford reagent to make final volume of 1ml. In these reaction mixtures, 0.1 µg/ml of BSA stock solution was used.

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

Table 3.6 Reaction mixtures for BSA standard curve

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.

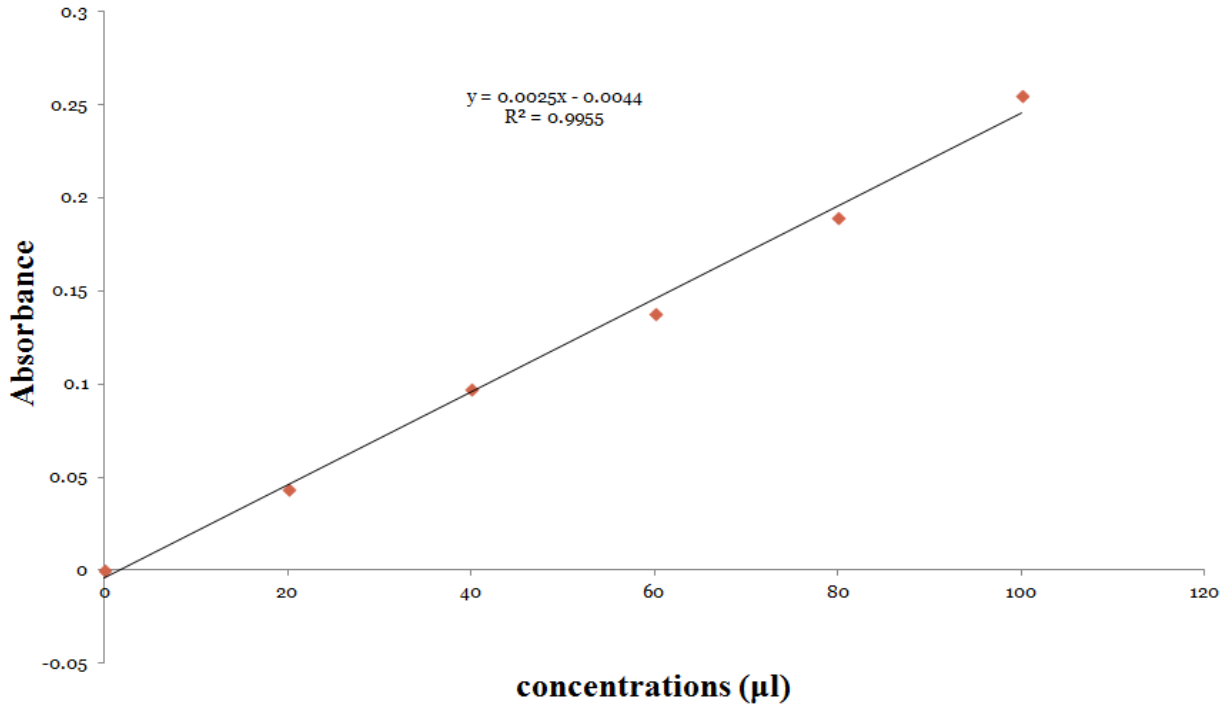

Figure 3.6: BSA Standard Curve

From this standard curve, regression line with the value of R^2 =0.99 was used to quantify protein content in our samples. And it was used to calculate sample loading values for western blot analysis.

3.7 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 of the sample mixtures prepared in Eppendorf 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.

3.8 Western Blot Analysis

3.8.1 Sample Loading Values:

Equation obtained from regression line of standard curve was used to calculate values of loading samples as given in table 3.2

	Absorbance	**2µg**	**1µg**	**20µg**	**H2O**	**4X**	**Total**
Control	0.533	0.935	0.467	21.41	3.58	7	32
CCL4	0.866	2.129	1.064	9.39	15.60	7	32
CCL4 +LBDE	1.09	2.935	1.467	6.816	18.18	7	32

Table 3.8: Calculation and preparation of loading sample values

Above mentioned sample concentrations were mixed with 4X Buffer and denatured at 95ºC. Denaturing step consisted of 15 minutes and followed by centrifugation of 2 minutes.

3.8.2 SDS Polyacrylamide Gel Preparation:

Optimization of SDS gel preparation led to use of 12% gel protocol. SDS gel was prepared in two steps, first step consisted of preparation and setting of separating gel and second step was preparation and setting of second layer called stacking gel.

3.8.3 SDS PAGE Electrophoresis:

Prepared samples, broad range protein marker and loading buffer were loaded in 12% acrylamide gel with the help of loading tips. Gel was run in the presence of 1X running buffer for 120 minutes at 90V.

3.8.4 Gel Staining:

Gel staining was carried out using commercially made gel staining solution called coomassie blue to visualize protein bands. This staining was done to confirm presence of protein content in samples. Proteins were distributed on gel in the form of bands according to their weight in the form of bands.

3.8.5 Gel to NC membrane 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.

3.8.6 Ponceau 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.

3.8.7 Blocking with non-fat milk:

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.

3.8.8 Primary Anti-bodies:

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

3.8.9 Treatment with Primary Anti-bodies:

After blocking step, NC membrane was treated with primary anti-body. This was done by shaking NC membranes dipped in prepared primary anti-body solution overnight or 18 hours at 4^{o}C.

3.8.10 Washing with TBST:

Right after completion of previous step, 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 in order to avoid false detection.

3.8.11 Secondary Anti-bodies:

Secondary anti-bodies were diluted in 1X TBS solution in 1:2000. Secondary anti-bodies were of rabbit, mouse and goat. Use of particular secondary anti-body was made according to the source of primary anti-body.

3.8.12 Treatment with Secondary Anti-bodies:

After washing step, NC membrane was incubated in secondary anti-body solution for 2 to 3 hours at 4ºC. While incubation time, gentle but continuous shaking was insured by commercially designed shaker. This step was also followed by washing with 1X TBST solution for 5, 5, 10, 10 and 10 minutes.

3.8.13 Particular Protein Bands Detection:

Final Step was to detect bands of protein of interest on NC membrane. NBT substrate purchased from Sigma Aldrich was used directly to detect protein of interest. Right after washing, NC membrane was exposed to 500µl of liquid NBT substrate. Treatment with NBT substrate caused protein bands to appear.

4. RESULTS

4.1: Anti-oxidant activity of Lirioresinol B Dimethyl Ether:

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 anti-oxidant activity was increasing with increase in concentration, as shown in figure 4.1. 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%.

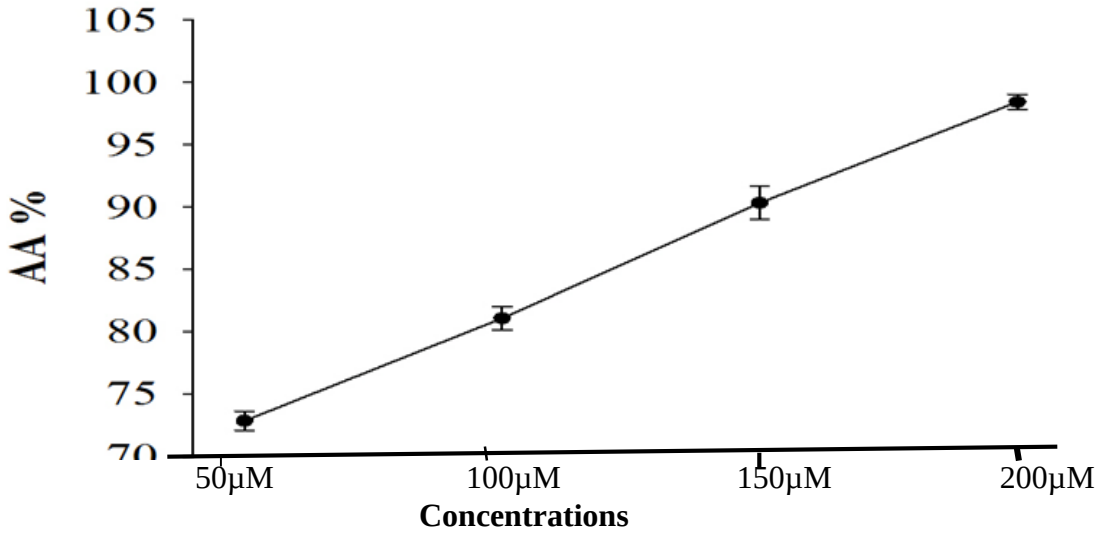

Figure 4.1: Percentage Anti-oxidant Activity (%AA) of Lirioresinol B Dimethyl Ether at different concentrations.

4.2: Liver Function Tests:

Two Liver Function Tests were performed, Alkaline Phosphatase (ALP) and Alanine Aminotransferase (ALT). Results shown that, levels of both ALT and ALP were increased to three fold when treated with CCL4 and were reduced back near to normal range when treated with Lirioresinol B dimethyl Ether.

LFTs	Normal Range	Control Group	CCL4	CCL4+LBDE
ALT	7 to 56 U/L	25 U/L	146 U/L	42 U/L
ALP	44 to 147 U/L	115 U/L	681 U/L	133 U/L

Table 4.1: Comparison of ALT and ALP levels among Control, CCL4, and CCL4+LBDE groups

For statistical analysis, One Way ONOVA was performed and comparison among experimental groups was strongly significant for both tests ALP and ALT, $P < 0.01$. ALP graph is shown in figure 4.2.1 and ALT graph is shown in figure 4.2.2.

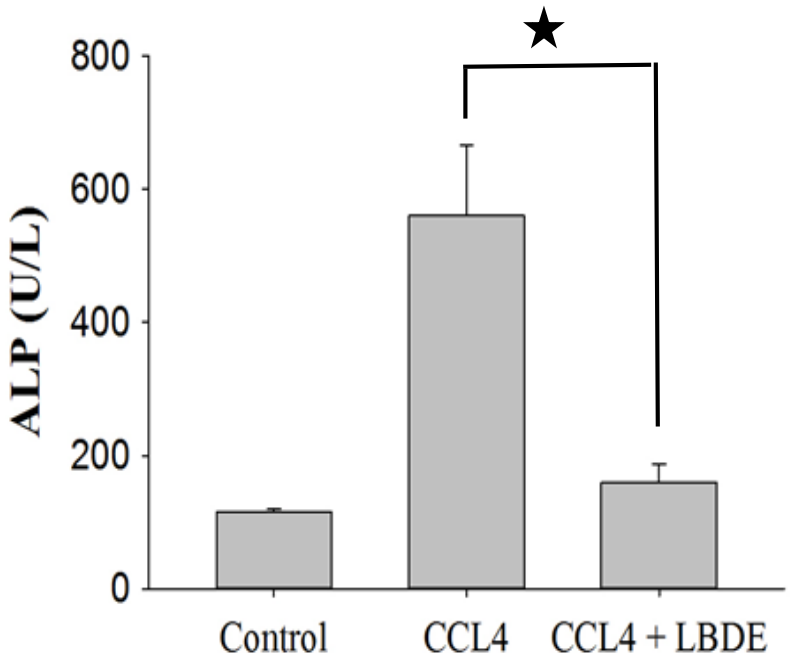

Figure 4.2.1: ALP test results comparison between experimental groups control, CCL4, and CCL4+LBDE. One way ANOVA test was performed.

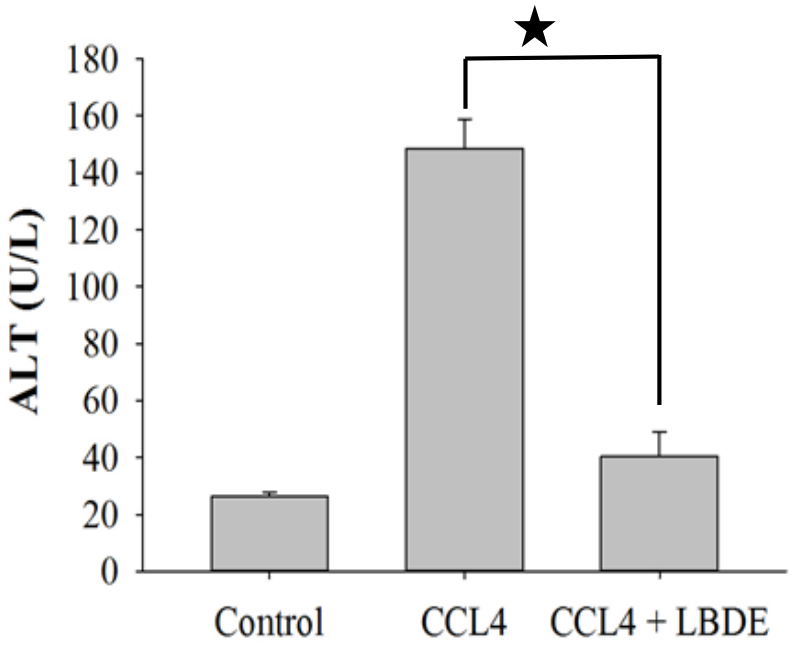

Figure 4.2.2: ALT test results comparison between experimental groups' control, CCL4, and CCL4+LBDE. One way ANOVA test was performed.

4.3: Histopathological Results:

Histopathology of liver tissues was performed for morphological examination. This was done by H&E staining and results were compared among all experimental groups, control, CCL4 treated and CCL4+LBDE treated. Results are shown in figures 4.3.1, 4.3.2 and 4.3.3. Figure 4.3.1 shows histopathology of control group, figures 4.3.2 a, b show histopathology of CCL4 treated group while figure 4.3.3 show histopathology of CCL4 + LBDE treated group.

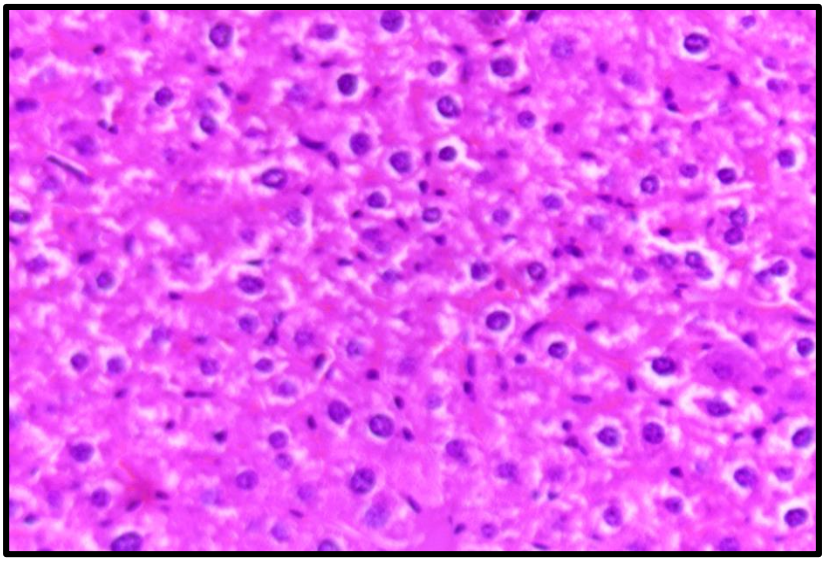

Figure 4.3.1: Histological examination of the section reveals hepatic tissue with normal nodal architechture. No granulomas are seen. There is no evidence of malignancy.

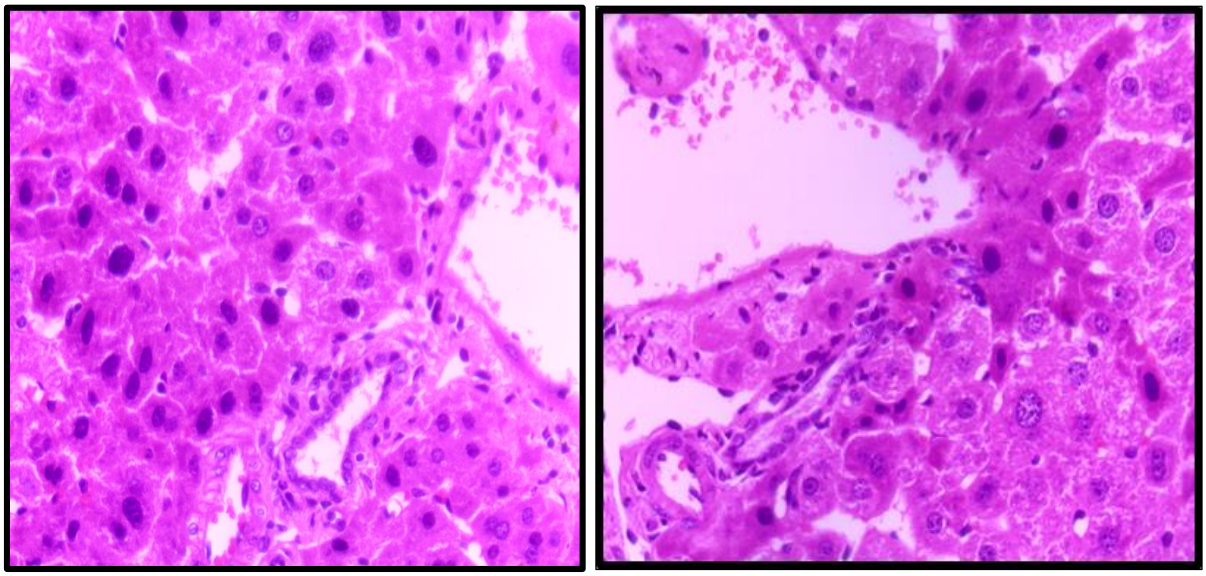

Figure 4.3.2 A and B: histopathology of liver tissue shows abnormal morphology of liver cells. Hepatocytes are arranged in sinusoidal architecture. Evidence of severe portal tract inflammation with highly dysplastic cells and congestion.

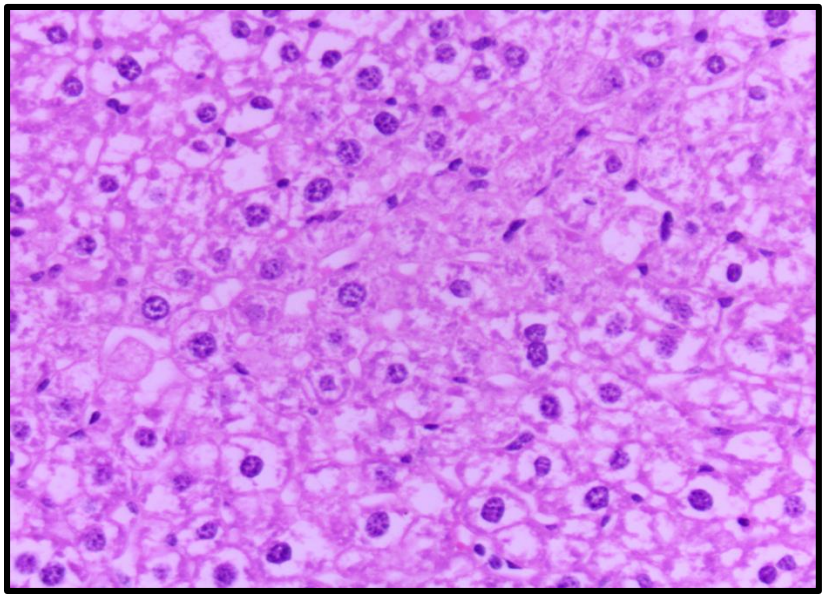

Figure 4.3.3: Histological examination reveals hepatic tissue with effacement of normal architecture. There are areas of congestion hemorrhage. Areas showing clearing arounds cells.

These figures show that liver cells of control group had normal architecture and with no marked inflammatory signs, while hepatocytes of CCL4 treated group of mice were highly dysplasitic and presence of inflammatory cells were observed mainly around portal tract. Lirioresinol B Dimethyl ether reversed inflammatory and dysplastic condition of liver cells as shown in histopathological figures, LBDE treated mice group hepatocytes show normal architecture with no signs of inflammation and dysplasia.

4.4 Western Blot Analysis:

Westen blot analysis as disscused in previous chapter was carried out to qualitatively analyse particular protein expressions. Results of Western Blot technique were visualised at three different stages, namely 1) Gel Staining for presence of protein bands, 2) NC staining with

Ponceau solution for detection of proper protein transfer from gel to NC membrane, and 3) final detection was carried out for particular protein expression levels.

4.4.1: Gel staining:

Gel Staining is done to confirm that samples contain protein content consisting of different cellular proteins that are separated according to their molecular weights in the form of bands as shown in figure 4.4.1.

Figure 4.4.1: Protein bands on SDS PAGE Gel.

4.4.2: Staining with Ponceau:

Ponceau staining again aids in visualising protein bands, but this time on NC membrane. Ponceau staining also confirmed that our protein of interest was present.and transfer step was properly done, as shown in figure 4.4.2.

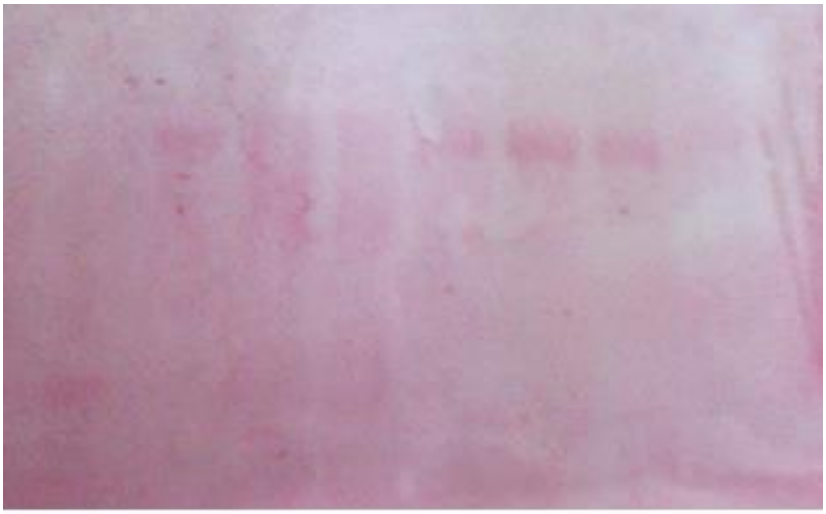

Figure 4.4.2: Ponceau Staining of NC membrane for protein detection

4.4.3: Final Protein Detection:

In this study, expression of NF-kB, I-kB and actin proteins were analysed and compared among all of the experimental groups control, CCL4 treated and CCL4 + LBDE treated groups. For this, NC membrane after ponceau staining and blocking with non-fat milk was treated with Primary anti-body (particular for protein of interest), secondary anti-body. Finally, NBT substrates was used for signal detection of protein of interest. In this way, proteins of interest appeared in the form of thick and thin bands depending upon their level of expression as shown in figure 4.4.3.

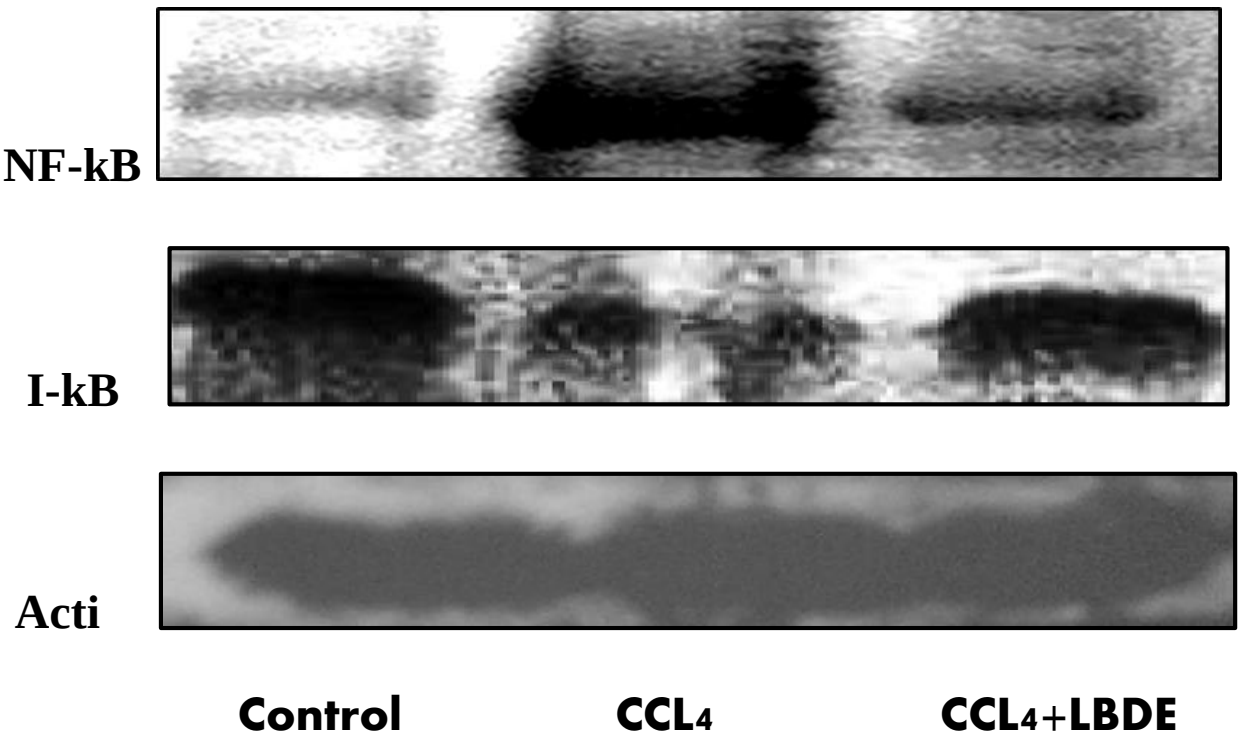

Figure 4.4.3: Protein expression in western blot results

Control group was left untreated, thus protein expressions of control showed normal protein levels. Western Blot protein expression levels reveal that, NF-κB was normally expressed in control group, wheeated with a carcinogenic substance ccl4, NF-κB was increased in inflammatory condition and cell dysplasia. NF-κB is key inflammatory regulator and its expressions are increased in inflammatory conditions. But when treated with Lirioresinol B Dimethyl Ether, NF-κB expression level was reduced back significantly.

Similarly, I-κB was being expressed in control group in required expression levels while, it was diminished when treated with CCL4, presenting inflammatory condtion in liver cells. Upon

treatment with Lirioresinol B Dimethyl Ether, I-κB expression levels was restored back showing anti-inflammatory activity of Lirioresinol B Dimethyl Ether in liver cells.

Actin expression levels remained same in all groups as it is muscle protein and is expressed normally and its expression is detected as a loading control.

5. DISCUSSION

Chronic Inflammation is known as body's failure to eliminate inflammation causing factors. Chronic inflammation is associated with diseases like cancer. It is reported that, one of the major cause of tumor development is chronic inflammation and second being the exposure of carcinogen. Chronic liver inflammation develops into third most common type of cancer called Hepatocellular carcinoma. In addition to this, research studies have outlined the whole pathway comprised of distinct phases namely hepatitis (liver inflammation), dysplasia, nodules and carcinoma (Pikarsky et al., 2004).

In this study, Lirioresinol B Dimethyl Ether was used to inhibit CCL4 (carcinogen) induced chronic inflammation and liver cell dysplasia to prevent cancer. Its anti-inflammatory role is reported against bone destruction. It inhibits chronic inflammation by downregulating Receptor activator of NF-κB Ligand (RANKL), to stop inflammatory vicious cycle (Jun et al., 2014). Lirioresinol B Dimethyl Ether was found to downregulate key inflammatory protein NF-κB in experimental group treated with Lirioresinol B dimethyl ether after CCL4 induced chronic liver inflammation. 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 2004), (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 (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.

Results of this research study revealed that, Lirioresinol B Dimethyl ether significantly inhibited chronic hepatitis by down-regulating NF-κB and up-regulating I-κB expressions levels in hepatocytes. Lirioresinol B dimethyl ether is first time studied for its role in liver injury and it prevented cancer development by inhibiting chronic liver inflammation and liver cell Dysplasia.

6. CONCLUSION

This study can be concluded from the results obtained that Lirioresinol B dimethyl ether regulates NF-κB/I-κB pathway to inhibit inflammation and prevent cancer initiation and progression. Lirioresinol B Dimethyl Ether inhibits NF-κB expression and its nuclear translocation to prevent transcription of anti-apoptotic proteins and inflammatory genes e.g., COX-2 and MMPs etc. Lirioresinol B Dimethyl Ether up-regulated expression levels of I-κB by inhibiting its phosphorylation and degradation, thus preventing NF-κB activation.

Lirioresinol B Dimethyl ether significantly inhibits chronic liver inflammation and liver cell Dysplasia and prevents cancer development by regulating NF-κB/I-κB inflammatory pathway.

Diagrammatic illustration to conclude this study is shown in figure 6.1

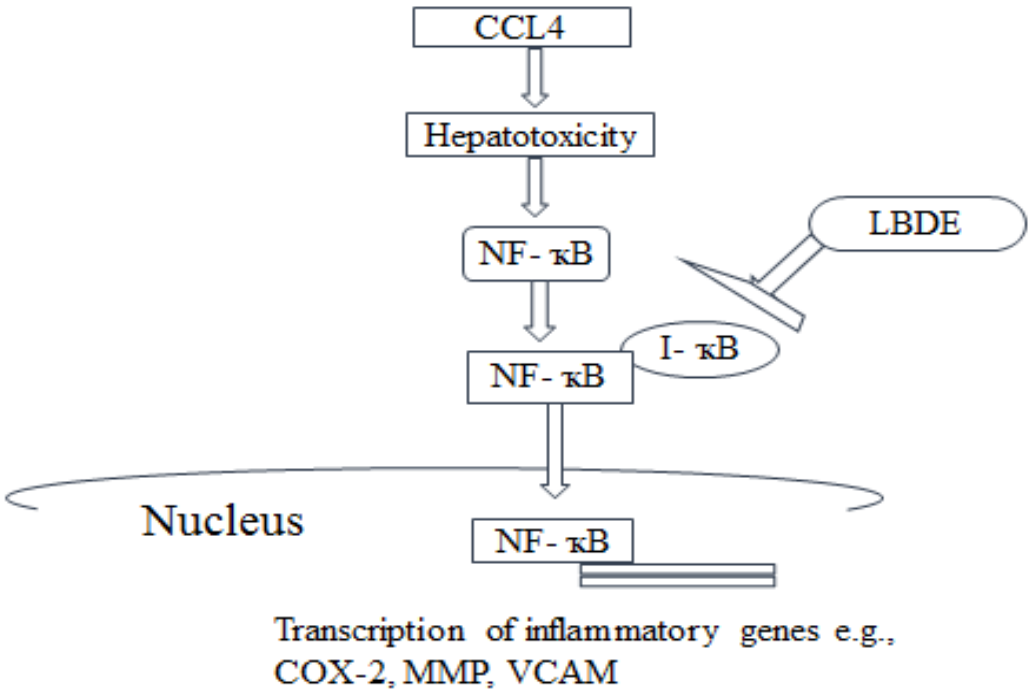

Figure 6.1: Mechanism of Action of Lirioresinol B Dimethyl Ether against chronic liver inflammation and Liver Cell Dysplasia

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Proposed Certificate for Plagiarism

It is certified that MS Thesis Titled **"Lirioresinol B Dimethyl Ethers Inhibits CCL4-induced Chronic Inflammation and Cell Dysplasia by Preventing NF-kB Expression"** by **Sajida Parveen** has been examined by us. We undertake the follows:

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