Lepidium pinnatifidum remediate CCL_4 induced oxidative damage in liver by regulating ER stress markers, inflammatory mediators and anti-apoptotic markers in rat.

A thesis submitted in fulfillment of requirements for the

Degree of Master of Philosophy

In

Biochemistry/Molecular Biology

By

Aemin Tahir

Department of Biochemistry

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In The Name of Allah

The Most Beneficent, the Most Gracious,

The Most Merciful

"And in the earth are tracts and (Diverse though) neighboring, gardens of vines and fields sown with corn and palm trees growing out of single roots or otherwise: Watered with the same water. Yet some of them We make more excellent than others to eat. No doubt, in that are signs for wise people."

(Sura Al Ra'd, Ayat 4)

Dedicated

To

My Parents

&

My Brother (Kaab Tahir)

Who taught me to put my trust in ALLAH, believe in my abilities within and that hardwork really pays off.

DECLARATION

I hereby declare that the work presented in the following thesis is my own effort and the thesis is my own composition. No part of this thesis has been previously presented for any other degree

Aemin Tahir

CERTIFICATE

This thesis, submitted by **Ms. Aemin Tahir** to the Department of Biochemistry, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad, Pakistan, is accepted in its present form as satisfying the thesis requirement for the Degree of Master of Philosophy in Biochemistry.

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AEMIN TAHIR

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List of abbreviations

Abbreviation	Full name
µg	Micro gram
µL	Micro liter
^0C	Centigrade
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
ATF	Activating Transpiration Factor
ALP	Alkaline phosphatase
BiP	Binding immunoglobulin protein
CAT	Catalase
CHOP	C/EBP homologous protein
CCl_4	Carbon tetrachloride
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
EDTA	Ethylenediamine tetraacetic acid
Elf2a	Eukaryotic initiation factor 2 alpha
ER	Endoplasmic Recticulum
G	Gram

GSH	Glutathione
GPx	Glutathione Peroxidase
GR	Glutathione Reductase
H_2O_2	Hydrogen peroxide
IL	Interleukin
i.e.	That is
i.p	Intraperitoneally
IC50	Inhibiton concentration
LPA	*L.pinnatifidum* aqueous
LPB	*L.pinnatifidum* butanol
LPC	*L.pinnatifidum* chloroform
LPE	*L.pinnatifidum* ethylacetate
LPH	*L.pinnatifidum* hexane
LPM	*L.pinnatifidum* methanol
Mg	Milli gram
mL	Milli liter
mM	Milli molar
Nm	nanometer
O^{-2}	Superoxide

OD	Optical density
OH^-	Hydroxyl ion

Abstract

Medicinal plants comprised of several bioactive chemical constituents which are providing significant compounds directly or indirectly which has proved to be an effective source to face the current healthcare challenges including cancer, infectious disorders, cardiovascular diseases, neurodegenerative diseases and several other types of lethal ailments. *Lepidium pinnatifidum* one of significant therapeutic plant which is traditionally used as a medicine in treating diseases like pile, constipation, menstrual pain, diarrhea etc. The pertinacity of our research study is to examine the hepatoprotective potential of *Lepidium pinnatifidum* against CCl_4.

Whole plant of *Lepidium pinnatifidum* was dried with crude methanol and then its fraction were made in several solvents in ascending order of polar activity starting from n-hexane (LPH), then chloroform (LPC), then ethyl acetate (LPE), and then butanol (LPB) and in the last aqueous fraction (LPA).

Qualitative analysis of *L. pinnatifidum* verifies the existence of phenolic and flavonoid content, also validates the occurrence of saponins, cumarins, alkaloids, tannins and glycosides. Terpenoids are absent in all fractions except in hexane fraction.

Hepatoprotective potential of hexane fraction *Lepidium pinnatifidum* was evaluated against oxidative damage in liver of rats mediated by carbon tetrachloride. At three levels; biochemical, histological and molecular level; liver tissues were studied. Treatment with plant fraction (LPH) in comparison to oxidative stress mediated by CCl_4 was provided to rats on alternate days for four weeks. Amount of glutathione (GSH) and antioxidant enzymes (CAT, SOD, POD) activity in liver were decreased in CCl_4 group in comparison to group treated with plant extract. However, in the case of peroxidation (TBARS) reduced level were present in group with plant treatment while CCl_4 group has relatively enhanced levels. Likewise, H_2O_2 and nitrite levels were markedly repressed in group receiving plant treatment in comparison to CCl_4 group. Rats with plant extract treatment showed restorative effects against CCl_4 toxicity in dose dependent manner. These results were additionally validated by the microanatomical study. Histopathological analysis revealed hepatoprotective potential of LPH with improved anatomical structures at high dose (400 mg/kg), which was more

productive in reducing the intoxications caused by CCL_4 as compared to low dose (200 mg/kg).

At molecular level, hepatoprotection from CCL_4 induced toxicity in liver tissues was also studied. As CCL_4 is one of the potent hepatotoxin, it has caused prolonged Oxidative stress which leads to ER stress, inflammation and if given for longer period of time apoptosis can occur. In order to study the hepatoprotective effect of plant extract ER stress markers, Inflammatory mediators and anti-apoptotic markers were studied. In groups treated with CCL_4 their expression level was elevated except ani-apoptotic gene whose level was depressed. But, in the rats with plant treatment the results were in opposite direction i.e. expression level of all the genes were reduced and in Bcl-2 it is increased close to the normal thereby elaborating the protective potential of plant extract.

The results elucidate that *Lepidium pinnatifidum* is a medicinal plant consisting of both antioxidant and hepatoprotective nature. Besides being a prominent antioxidant plant, it also illustrate promising activities like anti-microbial, anti- inflammatory and also anti-depressant activity due to the presence of large numbers of phytochemicals. Hence, the current study assures the therapeutic significance in Ayurvedic therapies, which could be the durable support to the advancement of new drugs.

INTRODUCTION

Plants have been utilized for so many years to give flavor and preserve food, to cure certain ailments and to avoid infections. The information about the therapeutic characteristics of plants has been spread out all over the globe among different nationalities (Silva and Fernandes, 2010). Cure with medicinal plants goes way back to the history of mankind. From several sources, the connection between man and his quest for medicines in nature lies in the far past about which sufficient evidence has been found in different composed records. Attention to therapeutic plants utilization is an aftereffect of the numerous long periods of battles against diseases because of which man figured out how to search for medications in barks, seeds, organic products, and different parts of the plants. Contemporary science has recognized their dynamic activity, so, medicinal plants have been incorporated into present-day pharmacotherapy. The awareness of the use of plants as a therapeutic agent and the advancement of medicinal plants in pharmacotherapy has helped the pharmacists and doctors to react to the difficulties that have developed with the spreading of expert administrations in assistance of man's life (Petrovska, 2012)

1.1. Importance of medicinal plants in the world and in Pakistan

Medicinal plants play a very vital part in the human medicinal services. Approximately 80% of the total world population depends on the prescription of natural drugs obtained from various products of medicinal plants (WHO 1993). Annually about 20% of the market for Ayurvedic medicines is estimated to be mounting in India (Subrat, 2003), while the number of medicinal plants got from only one area of China (Yunnan) has developed by multiple times in the most recent 10 years (Pei, 2002). Pakistan has several climatic zones and a variety of biodiversity in flora and fauna. It has around 6000 different types of higher plants present. It has been found that 600-700 plant species are medicinal in nature and have been utilized greatly for this purpose (Shinwari, 2010).

1.2. Brassicaceae, the crucifers family

Brassicaceae is the biggest family inside the Brassicales plant range. They incorporate trees, bushes, and herbs that are widely spread over from the Mediterranean area to

southwestern and central Asia and North America. This family contains around 4,000 species with 400 genera and incorporates numerous vital economic plants utilized both as edible and seasoning source. Arabidopsis thaliana (thale cress) which belongs to this family is one of the most commonly used experimental plant in biology (Koch *et al.*, 2006; Al-Shehbaz *et al.*, 2006; Judd *et al.*, 2002)

1.3. Ethnobotanical studies of genus *Lepidium*

Genus Lepidium (family Brassicaceae) consists of more than 175 herb species universally, commonly known species are peppercress, peppergrass, and pepperwort showing their sharp flavors (Radulovic *et al.*, 2008; Mummenhoff *et al.*, 2004). *Lepidium Meyennii,* commonly known as Maca is a product having its origin in the Central Andes of Peru. It is resilient to harsh conditions such as extreme cold and lengthy drought periods (Sifuentes-Penagos *et al.*, 2015). It not only has nutritional properties but it also is antioxidant and anti-cancer in nature. Therefore, it is widely used in the treatment of sexual dysfunction in both the genders(Ernst *et al.*, 2011). *Lepidium draba* commonly known as jangli meely is also a member of this genus. Its seeds are used as carminative and flatulence (Ullah *et al.*, 2013). *Lepidium sativum* is an important specie of Lepidium genus. Its common name is garden cress which is a very rapidly growing herb found commonly in Egypt and West Asia, but it is now grown worldwide due to its medicinal properties. It is a source of proteins, dietary fiber, iron, and some essential nutrients and phytochemicals. It is also used in medicines for the treatment of respiratory disorders including cough, asthma, and bronchitis. Its seeds are also used to treat inflammation, muscular pain, and rheumatism (Doke and Guha, 2014). *Lepidium latifolium* whose vernacular name is Chunma is another member of Lepidium genus. Its leaves infusion is used to treat liver diseases and its root powder is used to treat stomachache and kidney disorders (Bano at al., 2014). *Lepidium virginicum* which has a common name, virginia pepper weed or pepper grass. It is used as an anti-amoebic agent in the traditional Mexican therapeutics and used in treating diarrhea, mesentery, abdominal and epigastric pain, liver and renal diseases and also in worm infections. It is also used to treat anxiety and depression (Guzmán Gutiérrez *et al.*, 2014)

1.4. *Lepidium pinnatifidum* Ledeb.

Lepidium pinnatifidum is a perennial herb present in Lepidium genus. It is 50 cm in height with a solitary stem which is normally expanded. This stem contains thin hair which is short and simple. Basal leaves of the plant are vaguely dissected and sub-pinnatipartite. These leaves are slightly petiolate and dry early. Cauline leaves are generally elliptical and dentate,2-5cm in length. Upper leaves are relatively whole toothed, linear, oblanceolate or sub spathulate. Sepals are elliptic-lanceolate or ovate, petals are usually rudimentary or shorter than the sepals, number of stamens are two. Fruiting is racemes instead of loose and is with short thin hairs. Pedicels of this plant are thin and hairy. They are slightly longer and relatively more expanding in nature. Seeds are narrowly ovate, flattened, and elliptic, dark yellow in color, the flowering period is May- June, and fruiting in June (HAZRAT *et al.*, 2016)

1.4.1. Geographical distribution

L. pinnatifidum has been stated to be present in many countries globally. It is lately reported for the very first time in the flora of Korea (Hong et *al.*, 2016) and Turkey (Ünal *et al*, 2007). In Pakistan, it is found in Islamabad (Ahmad, 2009), Khyber Pakhtoon Khawan in district Mansehra (Farooq *et al.*, 2017), Battagram (Haq *et al.*, 2012), Dir (HAZRAT et al., 2013) and Hanna lake (Ahmad and Yasmin, 2011). It is present in district Bagh of Azad Jammu Kashmir (Tanvir et al., 2014).

1.4.2. ethnobotanical nature of *Lepidium pinnatifidum*

Lepidium pinnatifidum also have nutritional properties. It is utilized as potherb and vegetable (Haq *et al.*, 2012). Its leaves are broiled as a vegetable. *Lepidium pinnatifidum* is also used in traditional medicine. Its seeds are used to treat menstrual cramps in females and the whole plant is considered to be effective in pile and constipation (Mahmood *et al.*, 2012).

Figure 1.1: Herbarium specimen of *Lepidium pinnatifidum* Ledeb.

1.4.3. Taxonomic Ranking

As stated by Integrated taxonomic information system, the taxonomic grade of *L. pinnatifidum* is illustrated as follow:

Kingdom	Plantae
Subkingdom	Viridiplantae
Infrakingdom	Sterptophyta (land plants)
Superdivision	Embryophyta
Division	Tracheophyta (vascular plants
Subdivision	Spermatophytina (seed plants

Class	Magnoliopsida
Superorder	Rosanae
Order	Brassicales
Family	Brassicaceae (mustard family)
Genus	*Lepidium*
Specie	*Lepidium pinnatifidum* Ledeb
English name	Feather leaf pepper weed
Common name in Pakistan	Halian

1.4.5. Synonyms

Lepidium fastigiatum Ledeb.

Leoidium neubaueri Rech.f.

Nasturium fastigiatum (Ledeb.) Kuntze

1.5. Phytochemicals; the chemical components of plants

Phytochemicals are natural compounds found in plants as in medicinal herbs, vegetables, and fruits which along with nutritional compounds and fibers proves effective in several ailments and provide with us such components which explicitly provide defense against diseases. These phytochemicals are assembled in two major classes (Krishnaia*h et* al., 2009) known as primary components such as amino acids, simple carbohydrates, proteins, and chlorophyll, etc, and secondary components comprising of alkaloids, essential oils, flavonoids, tannins, terpenoids, saponins, phenolic compound (Krishnaiah *et al.*, 2007; deoga *et al.*, 2005). Phytochemicals could provide health benefits as: reactants for metabolic reactions, acting as coenzymes of the reactions catalyzed by enzymes, as inhibitory agents in several enzymes catalyzed

reactions, absorptive agents that react to and eradicate unwanted components in the intestinal mucosa, ligands that mimic all types of receptors whether extra-cellular or inside the cell, scroungers of free radicals and intoxicated substances, they also increase the captivation and stabilization of crucial nutritional compounds, discriminatory growing agents for useful bacterial flora present in gastrointestinal tract, anaerobic respirational reactants for mutualistic bacteria resident in mouth cavity and gastrointestinal lumen and specific inhibitory agents of poisonous bacterium of intestine. These types of phytochemicals are terpenoids, phenolics, alkaloids (Hussain *et al.*, 2011). Many of these phyto-compounds have been reported to exhibit significant curative properties which includes pesticidal (Kambu *et al.*, 1982) bactericidal, antimycotic (Lemos *et al.*, 1990), laxative (Ferdous *et al.*, 1992), antispasmodic (Sontos *et al.*, 1998), antiplasmodial (Benoitvical *et al.*, 2001) and antioxidant (Vardar-unlu G *et al.*, 2003)activities etc.

1.6. Oxidative stress, an enigma to aerobic life

Oxidative stress is defined as an increased level of reactive oxygen or free radicals in the cells that results in damage to proteins, lipids, and DNA (Schieber and Chandel, 2014). Oxidative stress causes several ailments i.e. cancer, nervous disorders, atherosclerosis, hypertension, ischemia/perfusion, diabetes, acute respiratory distress syndrome, idiopathic pulmonary fibrosis, chronic obstructive pulmonary disease, and asthma (Birben *et al.*,2012). ROS acts as an oxidizing agent that is formed from oxygen (O_2). Much of the ROS are formed from superoxide ($O_2^{-}\cdot$), which is produced when oxygen is reduced by a single electron. However, it is soon converted by superoxide dismutases (SODs) into hydrogen peroxide (H_2O_2). mitochondria have 8 sites that can generate ($O_2^{-}\cdot$) (Sena and Chandel, 2012). In living organisms, ROS are generated by general metabolic reactions and biological factors, such as air contaminants or smoke from a cigarette. ROS are extremely reactive species which distort cell structures such as carbohydrates, nucleic acids, lipids, and proteins and also affect their functions. Oxygen consuming organisms contains antioxidant mechanisms, comprising of enzymatic and nonenzymatic antioxidants which are useful in combating the deleterious effects of ROS (Birben *et al.*,2012).

1.7. Plants; the auspicious antioxidants

Plants have effective enzymatic and non-enzymatic antioxidant systems to combat with the free radicals. Enzymatic systems contain SOD, catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR) (Chand and Dave., 2009), and non-enzymatic systems comprised of compounds like ascorbic acid, glutathione, proline, carotenoids, phenolic acids, flavonoids, tannins, etc (Fig. 1.2).

Natural production of non-enzymatic anti-oxidants can be because of two reasons. Firstly, plants have an innate ability to produce them due to their genetic make-up in order to carry out their general biological functions and to guard themselves against herbivores and pathogens. Secondly, as a response to several harsh conditions i.e. environmental stress plants develop the tendency of phytochemicals production.

Low molecular weight antioxidants are also formed by plants that include glutathione and ascorbic acid. They are synthesized inside the stroma of chloroplast and cytosol by donation of an electron from NADPH (Alscher *et al.*, 1997). These antioxidants act as reducing and oxidizing buffers by reacting with several components of cells affecting growth and advancement of plants by modifying the processes like mitosis, cell elongation, senescence and death (Foyer, 2005). Moreover, to enhance defense these antioxidants may affect the expression of genes that are linked with the environmental stress response. During metabolism in aerobic conditions ascorbic acid (Vitamin C) is formed. As soon as its formation, ascorbic acid reacts chemically with oxygen radical, singlet oxygen and ozone and enzymatically with hydrogen peroxide via an enzyme; ascorbate peroxidase to nullify the noxious effects of these chemicals. One of the functions of Ascorbic acid is the regeneration of pigments with antioxidant effects (xanthophylls and carotenes), and also Vitamin E. Glutathione can both be active as oxidized and reduced form. It is present as GSH in reduced form and GSSG in oxidized disulfide form. It has some crucial functions in biosynthesis pathways, detoxification, antioxidant biochemistry and homeostasis maintenance (Meyer and Hell, 2005; Noctor *et al.*, 2012). Glutathione reductase enzyme converts GSSG into GSH by using the NADH as a reducing agent. GSH behaves as an antioxidant by reducing the level of ROS and it is also required in the ascorbate-glutathione cycle where it helps to remove harmful peroxides (Galant *et al.*, 2012). Plants synthesize vitamin E in the form of

tocopherols which act as significant buffer systems for lipid soluble substances. Tocopherols are formed in chloroplasts and protoplastids. In the cell, they are generally found in the membranes. It is one of the crucial singlet oxygen scroungers that helps in defending lipid peroxidation (Foyer, 2005; Jaleel *et al.*, 2009)

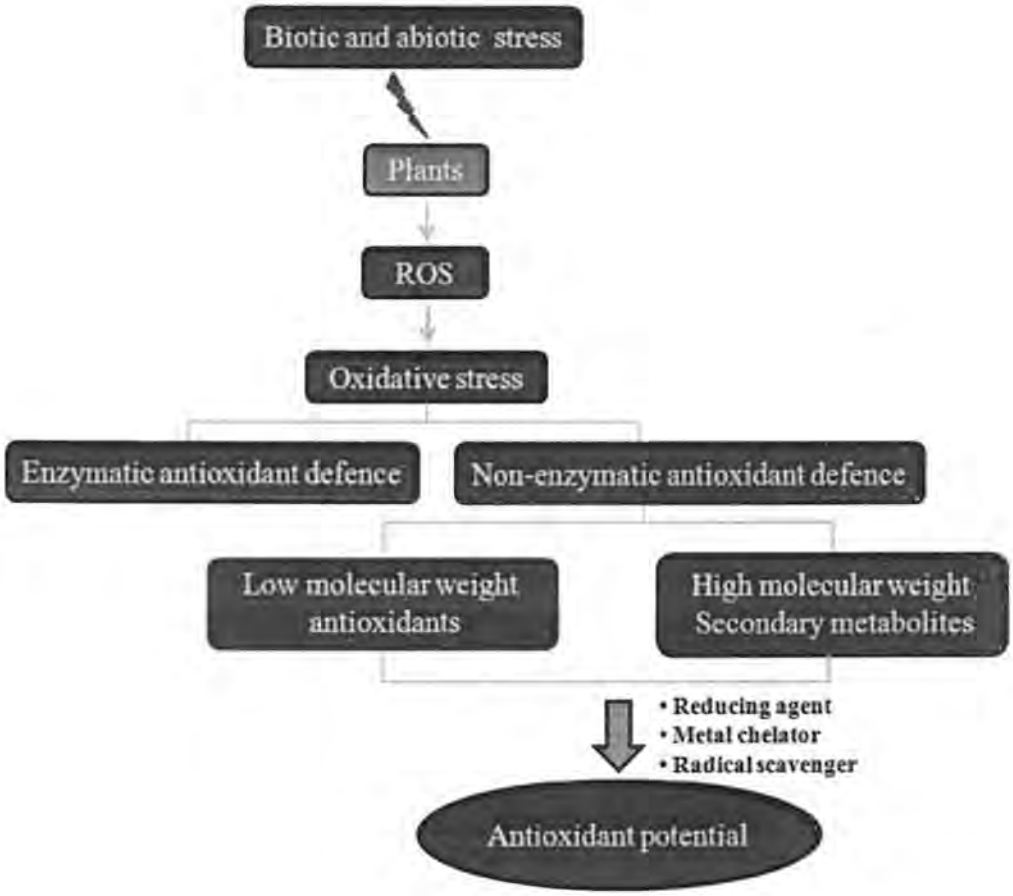

Figure 1.2. The antioxidant system in plants

1.8. Carbon tetrachloride; The free radical generators

Carbon tetrachloride is one of the most crucial hepatotoxin compounds of family haloalkanes (McGregor *et al.*, 1996). Due to the toxic effects of haloalkanes including carbon tetrachloride, chloroform, iodoform has been strictly prohibited for use. Although, still CCl_4 acts as a significant model to study the mode of action of hepatoxicity and its effects which includes fibrosis, lipid peroxidation, apoptosis of hepatic cells and hepatic cancer. Complete retrieval from hepatic damage can be

managed by managing the amount of dose used, the time of exposure to the hepatotoxin, the existence of accelerating chemicals having a synergistic effect with hepatotoxin and age of the organism.

Cytochrome (CYP)2E1, CYP2B1 or CYP2B2, and sometimes CYP3A activates CCl_4 which produces trichloromethyl radical, ($CCl_3^{\bullet}$). It can react with molecules of the cell including nucleic acid, protein, lipid. Upon binding with these molecules $CCl_3^{\bullet}$ impair the major cellular events like metabolism of lipid that results in degradation of fatty acids leading to steatosis. Binding of ($CCl_3^{\bullet}$) with DNA is considered as a trigger of cancer of the liver. ($CCl_3^{\bullet}$) produces the trichloromethyl peroxy radical which is quite reactive in nature upon the binding of CCl_4 with oxygen. $CCl_3OO^{\bullet}$ activates lipid peroxidation in a chain reaction, where it causes degradation of polyunsaturated fatty acids, especially the ones that form phospholipids of cell membranes which permeabilizes the membranes of mitochondria, endoplasmic reticulum, and plasma. It disrupts sequestering and maintenance of intercellular levels of calcium that greatly results in the cellular damage. Inhibition of significant enzymes occurs when a reactive aldehyde, 4-hydroxynonenal form bonds with certain functional groups of proteins. This aldehyde is formed as a result of the degradation of fatty acids. Hepatotoxicity of CCl_4 also causes demethylation of certain components of cells such as demethylation of RNA results in the dysfunctional protein synthesis and demethylation of phospholipid is thought to cause a decrease of lipoprotein secretion. None of these procedures, in essence, is viewed as a definitive reason for CCl_4 induced hepatic cell death, it is by participation that they accomplish a deadly result, provided this toxic substance works in a relatively high dose or over an extended period of time of low doses.

Activation of Tumor necrosis factor (TNF)α, nitric oxide (NO), and transforming growth factors (TGF-α and TGF-ß) takes place due to the action of CCl_4 intracellularly. These genes are involved in the signaling pathways that take cells towards apoptosis, necrosis as well as fibrosis. TNFα activates the death inducing signaling pathways and TGFs results in fibrosis. TNFα activates Interleukin (IL)-6 which is anti-apoptotic in nature, as well as IL-10 that acts oppositely to TNFα. Therefore, these interleukins

collectively have the ability to work up against CCl_4 to regain the normal function of hepatocytes.

By using several antioxidants and mitogens the above mentioned deleterious effects of CCl_4 can be reciprocated relatively by regaining methylation and restoring calcium sequestration. The molecules that activate cytochromes which are involved in the metabolism of CCl_4. As well as those chemicals that are given along with CCl_4 results in the delay of regeneration of hepatic tissue. So, they will regulate and increase the intoxication of CCl_4, while inhibitors of CYP450 can reduce the toxic effects of CCl_4 (Weber *et al.*, 2003).

1.9. ER Stress: Unfolded Protein Response

The endoplasmic reticulum (ER) has well-organized protein-folding machinery which consists of several proteins including chaperones, protein folding proteins, as well as indicators that sense misfolded and unfolded proteins. In ER there present a very accurate checking system that holds back misfolded protein from entering the pathways of secretion and guarantees that steadily misfolded proteins are coordinated towards apoptotic pathways. The unfolded protein response (UPR) is one of the homeostatic signaling pathways which is crucial in managing the homeostatic balance between folded and misfolded proteins and also helps in coordinating their need in ER (Malhotra and Koufman, 2007).

UPR occur when misfolded or unfolded proteins start to accumulate in endoplasmic reticulum which is detected by the sensor genes i.e. glucose-regulated protein 78 (GRP78) or binding immunoglobulin protein (BiP). As the misfolded proteins accumulate; it results in the sequestration of BiP. After the sequestration, BiP detaches itself from the three important ER-transmembrane transducers and in returns activates them. These include inositol requiring (IRE) 1a, PKR-like ER kinase (PERK), and activating transcription factor (ATF) 6a.

Eukaryotic initiation factor 2 alpha (eIF2a) is phosphorylated by PERK that results in attenuated mRNA translation, and also it counteractively translates various mRNAs; some of them are the transcription factor ATF4, and it's mediator CHOP which is present downstream. IRE1a contains both kinase and endoribonuclease abilities. Auto-

phosphorylated IRE1a causes activation of RNase action of splice XBP1 mRNA to form the sXBP1 which is the transcript variant. IRE1a also causes recruitment and activation of JNK which is activated in stress conditions. ATF6a moves to the Golgi bodies; here it is modified into its active form by cleavage through several proteases that are present in the inner membrane of Golgi. Some important roles of all these three UPR pathways are to build ER content, increase the protein folding ability of ER, eradicate unfolded or misfolded proteins, decrease the heap of new proteins that enter ER. These pathways are specially designed for the resolution and eradication of protein folding problems. Because of continuous ER stress, these pathways fail to perform their functions and as result apoptosis takes place. Apoptosis is caused due to the deregulation of calcium level, ROS generation and activation of proapoptotic gene CHOP (fig. 1.3.). In several hepatic disorders such as fatty liver disease, hepatitis caused by viruses and alcohol-mediated liver disease; activation of UPR commonly occur. All of these diseases are linked with steatosis, increasing the likelihood that steatosis is caused by the lipid peroxidation which occurs due to long term ER stress. Death of liver cells is a pathological condition in various hepatic disorders that might be associated with prolonged ER stress. If all of above statements have truth in them, then the resolution of ER normal function and management of ER stress-induced apoptosis can be a solid therapeutic target in hepatic disorders (Malhi and Kaufman, 2011).

1.10. Inflammation mediators

Inflammation is considered to be a necessary evil as it is one of an important innate immune response as well as hazardous for liver, influenced by various causative agents. Inflammatory responses that are mild in nature and could be easily resolved have reported showing continuous hepatoprotective results. These kinds of responses have contributed to the repairing of tissue damage and help in the restoration of liver metabolism (Brenner *et al.*,2013). Inflammatory mediators, mainly Interleukins family cytokines, Interleukin-6 (IL-6) and Tumor Necrosis Factor (TNF), are linked with chronic liver damage, the hepatic acute-phase response, steatosis, cholestasis, hypergammaglobulinemia, and fibrosis (Cressman, 1996). In the case of chronic liver injury, the above-mentioned cytokines not only cause liver cell death but also

regenerate the affected hepatocytes. Upon liver cell death IL-1α start releasing that causes the activation and recruitment of macrophages and bone-marrow-derived monocytes. Macrophages are also derived from the peritoneal cavity, and they are reported to have hepatoprotective nature. Some of the major causes of acute hepatic disorders are the secretion of TGF-ß as well as fibrosis. Prolonged inflammation and aggregation of fat molecules in the liver is the cause of alcoholic and nonalcoholic fatty liver disease. For managing these liver disorders antagonistic methodologies against inflammation have still not administered (Netea *et al.*, 2017)

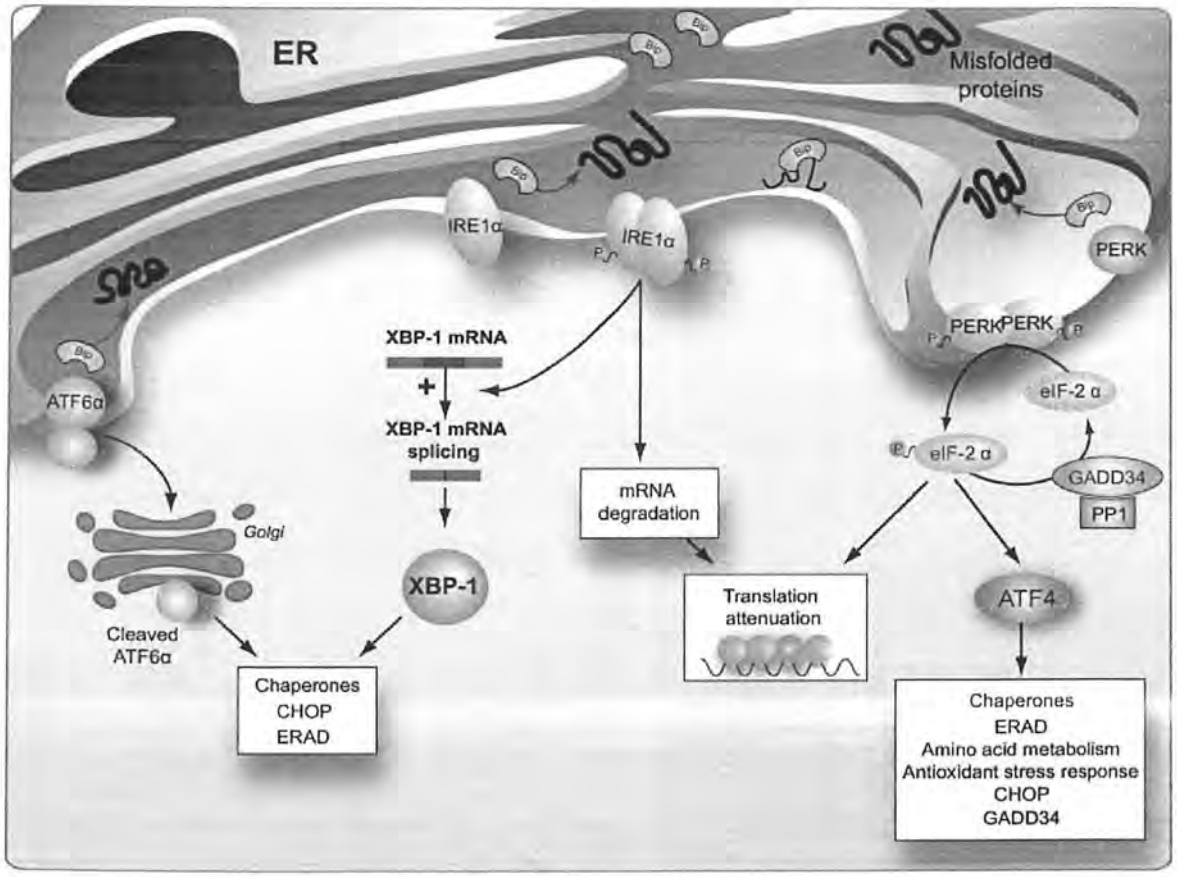

Fig. 1.3. ER stress pathway

1.11. Anti-Apoptotic markers

Apoptosis is a kind of programmed cell death, and any dysfunction in this systemic cell death would cause in certain diseased conditions which include autoimmune disorders, cancer, and neural disabilities. Major key components of apoptotic proteins are the Bcl-2 family members including pro as well as anti-apoptotic proteins. Any mild

disturbance in their management might become the cause of inhibiting or promoting apoptosis. Apoptosis is executed by the activation of both, intrinsic and extrinsic pathways. Once these pathways are activated, they result in the initiation of several downstream signaling events and caspases, and the release of several apoptotic entities from the mitochondrial membrane. All of these events are crucial in determining the fate of the cell (Ola *et al.*, 2011).

1.12. Liver fibrosis

As a consequence of prolonged hepatic damage, extracellular matrix (ECM) accumulate progressively which results in liver fibrosis. If the proliferation of fibrous tissue persists, the deposition of ECM modified the liver morphology resulting in the chronic liver failure, and increase in hepatic liver pressure gradient, and exaggerate other issues that complicate cirrhosis (Sancho-Bru and Ginès, 2016). Toxicity of hepatocytes results in hepatic cell death and hepatic stellate cells relocate to the site of damage to destroy the apoptotic bodies. Hepatic myofibroblasts are formed by activating hepatic stellate cells upon engulfment. Hepatic myofibroblasts in their active form endorse the accumulation of ECM and generation of the liver scar.

It has been reported that persistent fibrosis and apoptosis of hepatocytes takes place if these cells specifically become deprived of Bcl-xL (T. Takehara *et al.*, 2004). Certain inflammatory mediators such as IL-6, TNF-α also causes induction of chronic hepatic inflammation (A. Canbay *et al.*, 2003).

1.13. Aims and Objectives

The aim of this study is to determine the hepatoprotective potency of *Lepidium pinnatifidum* on CCL_4 induced liver hepatotoxicity.

1.14. Work Plan

Part I

Gathering, authentication, and drying of L.p*innatifidum pl*ant. To explicit the potential ability of L.*pinnatifidum,* production of crude extracts and making of fractionation for examining the active agents/molecule.

Part II

- In vitro qualitative analysis of different fractions of plant
- In vivo assessment of *L.pinnatifidum* against CCl_4 mediated hepatotoxicity in rats.
- Histological studies of the liver of rats.
- Elucidation of antioxidant enzymes activity including POD, CAT, SOD, GSH, TBARS, H_2O_2 and Nitrite assay in liver tissue homogenates. Serum examinations for liver biochemical markers i.e. ALP, AST, ALT, and total proteins.

PART III

- RNA extraction from liver tissues of rats.
- Qualitative and quantitative confirmation of extracted RNA
- cDNA synthesis from extracted RNA samples
- RT-PCR analysis to elucidate the expressions of ER stress markers, Inflammatory mediators and anti-apoptotic markers using synthesized cDNA.

Review of Literature

Since the origin of Earth with the feasible environment, mankind has been closely associated with it. He started to use the materials present in environment for his need such as he attained food and make medicines from plants. Knowledge and usage of plants to obtain food and medicinal drugs has been understood via awareness and application of plants to prepare food and medicine have been realized through series of experiments and tests, and in this way man became familiar with the use of environment to fulfill his needs. Knowledge of therapeutic plants started to transmit from one generation to the next progressively. This information has successfully achieved its goal when several societies were born along with establishment of services. From ages medicinal plants have been utilized as a source of therapeutic and medicinal drugs for every civilization and nation worldwide (Jamshidi *et al.*, 2018).In China, India , Egypt and Greece medicinal plants shared oldest place in history of science . In prehistoric time of Persia, medicinal plants have been generally utilized in the form of medicine, sterilizer and as a fragrance (Hamilton, 2004). Lately medicinal plants are one of the major topic in developed as well as in developing countries because of their safe use without much side effects, and effective nature.

Ethnobotany is the branch of ethnobiology in which we investigate and understand the information that man holds about the culture significance, is the study and interpretation of human knowledge about the cultural meaning, administration and folk utilization of floral parts of plants. Ethnobotany is also known to study the interrelationship among human being and plants (Pasa *et al.*, 2018).Since the ancient civilization; use of plants in the folk medicines have been adopted by people through the procedures of both ethnobotany and humanities that emphasize the ancient knowledge and its transmission among different nations (WHO 2002). A very large number of people still use several medicinal plants and their products for their wellbeing and regular health treatments. It is true that one from the every four prescribed medicines are made from the chemicals obtained from plants or they are the derivatives of plants products. WHO has reported almost 80 percent of the population of world chiefly comprising of developing countries

depends on plant based drugs (Gurib-Fakim, 2006). Some examples of the medicinal plants producing medicines are Digitalis spp, Cinchona spp, *Catharanthus roseus*, *Atropa belladon*a and *Papaver somniferum* producing digoxin, quinidine and quinine, vinblastine, atropine and morphine and codeine on large scale and demand. It has been reported that approximately 60% of anti-cancer and antibiotic, antifungal, anti-protozoan and antiviral medicines both on experimental trials as well as those that are available in market are derived from natural products .(Ullah *et al*., 2013).

By normalizing and assessing the protective effects of products derived from plants, medicines obtained from plant source can become an aid for the development of modern period of health services to cure several human ailments in near future (Jamshidi *et al*., 2018).

2.1. Contribution of genus Lepidium in therapeutic battle

Lepidium Linnaeus (1753: 643) is among the 3 biggest genera present in the family Brassicaceae (Al Shehbaz, 2012) . It is recently stated by (de Lange et al., 2013) that this genera contains nearly 260 species which are present all over the globe. Variety of different Lepidium species are distributed in Asia particularly in Middle, Central and South West Asia, forming Asia as one of the hub of diverse and complex nature of this genera (German, 2014).

This genus contains some important medicinal plants that have therapeutic properties. *Lepidium sativum* Linn (the garden cress) is commonly consumes as a salad and is grown all over in India now but this plant is not native plant of India. Garden cress is a rapidly growing herb which is eatable. It is known for its medicinal properties even in ancient vedic era. This plant have been reported to be used in treating number of diseases including asthma, cough, leucorrhoea, scurvy, diarrhea, dysentery, skin disorders, liver and kidney disease. *Lepidium sativum* is used as water pills, stimulant, abortion inducing drug, heat inducing agent, adjuvant, laxative, optometric agent (Raval, 2016).

Lepidium meyenii commonly called Maca is a plant member of Brassicaceae family grown since 18th century natively in Peru. It is distributed widely in the central Andes at an altitude 4000 and 4500 m high. This plant is edible in nature and is known for its therapeutic effects in folk medicines. Experiments and trials have proved that it acts as a nutrient, stimulator and fertility-enhancer. Maca has also been proved to be effective in prostate cancer, bone disorders, sterility, sexually transmitted diseases, protection of skin from UV radiation, development of memory and in learning process. Experimental tests have shown that Maca can be used to enhance the number of sperms and their movement. Clinically Maca has been reported to act as a is a plant with great potential as an adaptogenic herb and also as a nutraceutic to prevent various disorders (Gonzales, 2012).

Lepidium draba L which has common name 'hoary cress' is a perennial herb of Brassicaceae family. This plant is a native of western Asia primarily in Turkey, Iran and Armenia, In Central Asia and in coastal areas of Black and Mediterranean Sea of Europe. It was firstly found on the eastern coastal areas of North America in late 1800s. At present time Hoary cress is present in most parts of Canada, America and Mexico. *Lepidium latifolium L.* also called 'pepperweed' another significant member of *Lepidium* genus. It is found natively in Asia and Southern Europe (Francis and Warwick, 2008; Kaur *et al.*, 2013). Both of these herbs have been utilized by the local people differently for their own use. *L.draba* is widely distributed in certain areas of Iran where the soil is adequately moisturized and have diverse niches (Miri *et al.*, 2013; Shahrokhi *et al.*, 2014).It is used an ingredient in cooking rice in their folk foods (.and also act as chemopreventive, nutrient and pharmaceutical substance while Pepperweed is utilized as a vegetable in the form of salad, phytonutrient and also in energy drinks (Rana *et al.*, 2012; Kaur *et al.*, 2013)

2.2. Free radicals; the initiators of oxidative stress

Free radicals are produced during body metabolic processes naturally. It is known as a specie that is comprised of 1 or more than 1 unpaired electrons present in the outer most or valence shell and can exists independently. Because of the existence of these

singlet electrons; free radicals are unstable, forms only for a short time and radially reacts with other molecules. As free radicals are highly reactive in nature, they can possibly knock out electrons from other substances in order to make themselves stable. Therefore, the targeted compound becomes deficient in its electrons that renders it to change into a free radical too, starting a cascade that ultimately results in damaging the cells (Mukherji and Singh, 1984). Free radicals are in two forms: reactive oxygen species (ROS) and reactive nitrogen species (RNS). Both of them have internal and external origins. Endogenously they are obtained from endoplasmic reticulum, mitochondria, peroxisomes, phagocytes while exogenously from cigarette smoke, heavy metals, environmental pollutants, industrial wastes, certain metalloids, pesticides and several medicines and radioactive elements. Free radicals have adverse and deadly effects on several significant micro and macromolecules inside the body including DNA, RNA, proteins and lipids, where they cause alterations in general reduction and oxidation reaction directing towards oxidative stress (Phaniendra *et al.*, 2015). ROS and RNS constitutively form free radicals as well as other reactive species that are not radical (Pham-Huy *et al.*,2008).Some of the radicals species are Superoxide ($O^{-2}O^{-2}$), Oxygen radical ($O^{\cdot-2}O^{\cdot-2}$), Hydroxyl ($OH^{\cdot}OH^{\cdot}$), Alkoxy-radical ($RO^{\cdot}RO^{\cdot}$), Peroxyl radical ($ROO^{\cdot}ROO^{\cdot}$), Nitric oxide ($NO^{\cdot}NO^{\cdot}$) and nitrogen dioxide ($NO^{\cdot 2}NO^{\cdot 2}$) (Halliwell B, 2001). Free radicals are highly reactive because of the existence of a single unpaired electron. This electron can be donated to other molecule or a free radical can take up an electron to become stable. Examples of non radical species are hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), hypobromous acid (HOBr), ozone (O_3), singlet oxygen ($^1O_2$), nitrous acid (HNO_2), nitrosyl cation (NO^+), nitroxyl anion (NO^-), dinitrogen trioxide (N_2O_3), dinitrogen tetraoxide (N_2O_4), nitronium (nitryl) cation (NO_2^+), organic peroxides (ROOH), aldehydes (HCOR) and peroxynitrite (ONOOH) (Kohen and Nyska, 2002; Phaniendra *et al.*, 2015)

2.3. Antioxidant nature of medicinal plants

From the ancient time dietary antioxidants can be readily obtained from plants. It is reported that about two-third of the plants inhabited on Earth have therapeutic properties, and all of them have efficient antioxidant nature (Krishnaiah *et al.*, 2011).These antioxidant properties of plants have been a hot topic in healthcare system due to the extensive oxidative damage, which is the major cause of several deadly and infectious disorders like neurodegenerative and cardiovascular diseases. Moreover, use of dietary antioxidants as supplements and enhancing the level of endogenous antioxidants has been proved to be effective to combat the deleterious effects of stress caused by free radicals (Kasote *et al.*,2013)

There are several methods by which body forms number of oxidants in little proportions during metabolism. Free radicals are produced by neutrophils and leucocytes in small amounts when these cells are subjected to microbes which provides defense against the threat and therefore activate immune system to kill these invading microbes. But accumulation of oxidants in greater number can prove to be deleterious to human body. Liver is known to be one of the most affected organ due to oxidative stress and is greatly affected by the presence of hydrogen peroxide. After the continuous exposure of hepatotoxins to liver cells, lipid peroxidation can occur which is the major cause of liver pathologies. There are several substances already present inside the body and also certain exogenous substances have anti-oxidant properties. A lot of substances that are taken in diet as well as those that are already present in the body such as superoxide dismutase, ferritin, transferrin, reruloplasmin, tocopherol, carotene and ascorbate contains antioxidant and free radical foraging properties. Various whole plant and formulas obtained from them are commercially exist for this foraging action. Moreover, nowadays it is trending all over the globe to reanimate the traditional natural drugs and restored attention towards the cure and treatment present in nature for curing human diseases. Antioxidants possess several significant protective functions, it is used as a mean to give sensational modification in flavor and helps to increase the nutritive potential of certain food items. Along with these properties antioxidants can help revert the tissue injuries and several other human ailments. Approximately, all the living creatures have defensive mechanisms either in the form of enzymatic system or other

chemical components, i.e. ascorbate, α-tocopherol and glutathione to protect themselves from harmful effects caused by oxidative stress (Sharma *et al.*, 2013)
There are large number of medicinal plants which are reported to have antioxidant potential in nature. The Naringenin which is an oxidant that scavenger superoxide radicals, was reported to be present is the methane fractions of *P. sarmentosum and M. elliptica* leaves and known as prominent antioxidant used as a food item (Tsoyi et al., 2013). In Rosaceae family, *Rosa rugosa and Rosa davurica* expressed predominant radical-foraging properties. Medicinal plants that were proved to have maximum antioxidant potency from different families excluding Rosaceae, were *Cedrela sinensis* from Meliaceae family, *Nelumbo nucifera* of Nelumbonaceae, *Eucommia ulmoides* of Eucommiaceae, *Zanthoxylum piperitum* of Rutaceae, *Cudrania tricuspidata* of Carrière and *Houttuynia cordata* from Saururaceae family (Cho *et al.*, 2003).some significant antioxidants constituents are present in *Cissus quadrangularis and Muntingia calaura* which are known to be effective in case of diseases caused because of oxidative damage (More *et al.*, 2018).The antioxidant effect of flavoniods, is *Scutellaria baicalensis* Georgi is a member of Lamiaceae having antioxidant potential in its flavonoid compounds and is used in folk medicines in Japan and China . Some phytochemical components of S. *baicalensis* including baicalein, baicalin, wogonin act as an antioxidant, anti-inflammatory, antiplatelet, antibactericidal and antiviral (Broncel, 2007).

2.4. Mechanism of action of CCl_4

In lab based studies involving liver damage, carbon tetrachloride (CCl_4) is generally utilized as a hepatotoxin to generate chronic liver damage in model organism (Fujii et al., 2010). CCl_4 is absorbed by cytochrome P450 (CYP2E1) in liver to generate trichloromethyl and trichloromethyl peroxy radicals, that lead to the induction of oxidative stress, peroxidation of lipid molecules, and liver damage (Martinez et al., 1995) as shown in Figure 2.1 (Manibusan et al., 2007). Along with oxidative stress, inflammation is also one of the significant process interceding CCl_4-induced hepatic damage (Niu *et al.*,2017; Tacke *et al.*, 2009). In this mechanism, pro-inflammatory mediators involving cytokines and chemokines play key roles that causes apoptosis and

hepatic damage (Seo *et al.*, 2016; Shim *et al.*, 2010; Wasmuth *et al.*, 2010). Similarly, earlier studies have shown that hepatic damage can be averted by reducing oxidative stress and inflammation (Lin *et al.*, 2012; Tipoe *et al.*, 2010). So for this purpose, metabolic and inflammatory signaling pathways are considered as therapeutic targets in the treating hepatic agents. On contrary to this , hepatic injury and retrieval also include extracellular matrix remodeling, which is a process of structural modification that took place in each tissue, like liver (Massey *et al.*,2017; Poole *et al.*,2016).

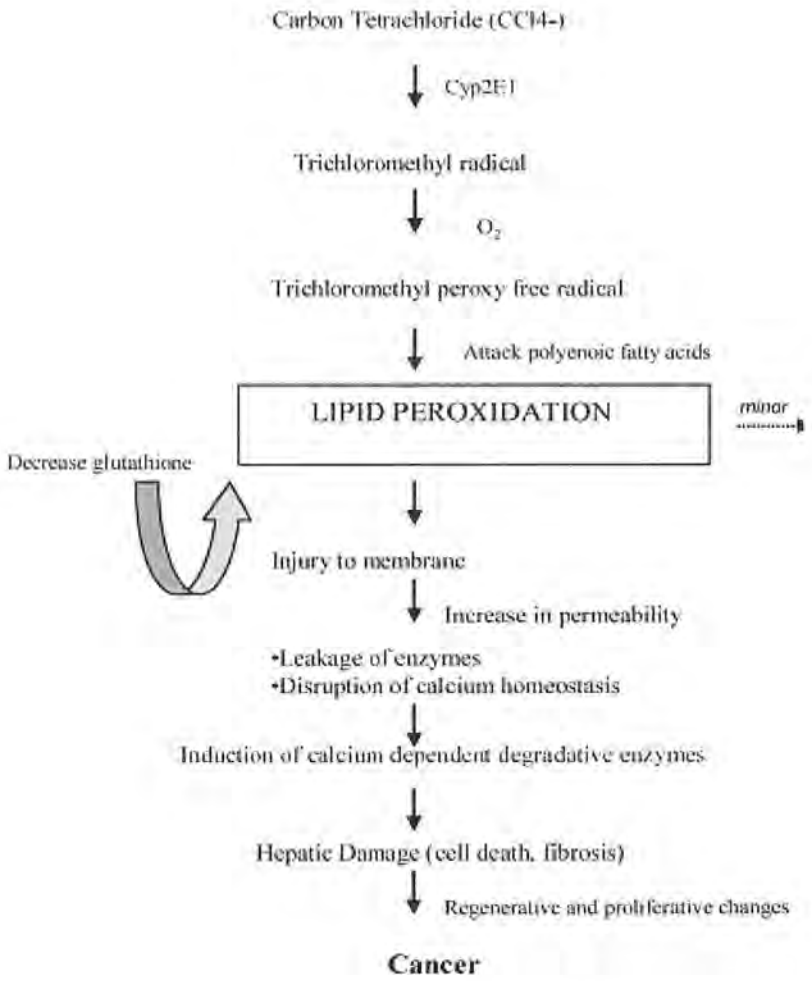

Figure2.1 Mode of action of CCL_4

2.5. Liver; the hub of metabolism

The liver is known to be the largest gland of body, weighs approximately 2–3% of body mass. There are three surfaces of liver namely diaphragmatic, posterior and visceral surfaces (Hu *et al.*, 2019). Weight of liver in males is about 1.4-1.6 kg and in females it weigh 1.2-1.4 kg (Lau et al., 2016). Liver is located between heart and the organs of gastrointestinal tract. The major role of liver is absorption and storage of nutrients. It is also involve in the provision of nutrients to other organs of the body. Along with nutrient uptake liver also take up the damaging entities.

Liver is considered to be one of the best and cheap recycling plant installed in the body of organisms. Liver comprised of parenchymal and non-parenchymal cells that take part in the recycling procedures. As liver take up the harmful substances, these substances tend to pile up inside the liver rendering damage to liver tissues. Therefore, precautions should be taken in order to prevent liver damage. Kupffer cells, sinusoidal endothelial cells are the members of non-parenchymal cells that are among the defense system of liver (Ramadori *et al.*, 2018).

2.5.1. Hepatotoxicity

Hepatotoxicity is known to be caused by the exposure of any substance on liver that leads to abnormality in its function, structure and infers chemical based, drug based and hepatic damage due to microbes (Subramaniam *et al.*, 2015). Liver injury is associated with the modifications in these roles of metabolism. Liver injury is linked with necrotic cell death, rise in peroxidation of lipids and reduction of diminished levels of glutathione. Moreover , levels of various biochemical markers in serum including Aspartate aminotransferase, alkaline phosphatase, bilirubin and cholesterol are increased in liver injury (WHO 1993).

Hepatotoxicity is classified into the following classes:

(a) Hepatocellular injury: Occur when ALT or ALP levels in serum are raised;

(b) Cholestatic injury: Occur when ALP and bilirubin serum levels are elevated;

(c) Mixed injury: Occur when ALT and ALP serum levels are elevated (Navarro and Senior, 2006).

Hepatotoxin is a known toxin composed of such chemical constituents that cause liver injury. Intoxicated hepatic damage generated by medicines and other substances can

generally imitate any kind of liver disorder that occur naturally. Several organic compounds have the potency to generate hepatotoxic, some of these compounds are iron, copper, phosphorus and arsenic. Natural organic products of some plants are toxic in nature like pyrrolizidine alkaloids, mycotoxins and bacterial toxins.

2.5.2 hepatoprotective effects of medicinal plants

Hepatoprotective potential of medicinal plants was evaluated when model organisms(i.e. mice or rats) were exposed to certain substances, medicines and toxins that toxicity in them including alcoholic products , carbon tetrachloride, galactosamine, paracetamol, isoniazid, rifampicin, peroxidised oil and aflatoxins etc (WHO 1993). Drugs obtained from medicinal herbs and whole plants are needed to treat different ailments and they are on demand on daily basis. Medicinal plants may be considered as one of the safest method to treat hepatotoxicity as they show minimum negative results and these plants also contain significant phytochemicals involving flavonoids, alkaloids, glycosides, that show antioxidant potency and hence proved to be effective in treating liver injuries. These are some of the examples of the plants that are hepatoprotective in nature, *Andrographic paniculata*, *Aegle marmelos*, *Allium sativum*, *Gymnemasylvestre*, *Pyrenthrum indicum*, *Taraxacum officinale*, *Berberis lyceum*, *Bryonia alba*, *Lycopersicon esculentum*, *Luffa echinata*, *Nigella sativa*, *Ocimum sanctum*, *Terminalia chebula Tinospora cordifolia*, and *Zingiber officinale* etc. all of these therapeutic plants have the potency to produce phytochemicals in significant amount that are used to treat hepatotoxicity (Fatima and Dhobi, 2018)

2.5.3 ER stress: consequence of liver toxicity

Carbon tetrachloride (CCl_4) is a transparent chemical which is present in small ratio in atmospheric air. When CCl_4 is orally administrated, it is accumulated in the liver. One of the characteristics of CCl_4 hepatotoxicity is the aggregation and peroxidation of lipids especially triglycerides in hepatic vicinity (Pan et al., 2007; Paz Gavilan et al., 2006; Weber et al., 2003). Assembling and releasing of apolipoproteins from liver is required for the secretion of triglycerides, that is a fundamental part of the endoplasmic reticulum (ER) functionality. Toxicity of lipids in liver occurs upon the inhibition of apolipoprotein B(ApoB) release; which is inhibited when ER becomes

nonfunctional(Pan et al., 2007; Hussain et al., 2003). It has been reported that CCl_4 administration lead swelling of rough ER in the cells of liver, resulting to express GRP78 and XBP-1, the essential markers of ER stress (Marumoto et al., 2008). ER stress has been detected primarily by the members of transmembrane proteins of ER including endoribonuclease IRE1, PKR-like ER kinase (PERK), and activating transcription factor 6 (ATF6). Unfolded protein response (UPR) is a collective series of extremely precise signaling cascades which is the innate defensive system of ER to combat against ER stress(Zhang and Kaufman, 2004). As soon as UPR activate, it causes specific ER chaperone proteins including GRP78 to upregulate, that enhances the folded protein concentration in ER as well as it inhibits the formation of aggregated proteins(Paz Gavilan et al., 2006). It has also been proved that the deposition of unfolded proteins inside the lumina of the ER can result in generation of UPR for a very long time, which results in diseased condition such as lipid deposition in liver (Sozio et al., 2010; Bowes et al., 2009). Pathological phenomenon can also occur due to the deposition and concentration of free radicals (ROS) as a result of ER stress activation for longer period of time.(Hayashi et al., 2005)

2.5.4 Hepatic inflammation; A silent killer of hepatocytes

As a response to several stress stimulus on liver, activation of a highly specific phenomenon takes place known as hepatic inflammation, which leads to several severe and long-lasting hepatic disease (Kubes and Mehal, 2012). Like in every organ, hepatic inflammation occur in liver to prevent hepatic cells from severe damage, to help in repairing the tissue injury, and to endorse the restoration of homeostatic nature, in short, providing constant hepatoprotection. But those inflammatory actions that are too strong or unable to restore take the shape of chronic nature, are constantly going to be escorted by an immense deprivation of hepatic cells and therefore become reason of the permanent harm to the parenchyma of liver (Schattenberg *et al.*, 2006). The dead hepatocytes then soon be replaced by the new ones when hepatic stem cells generate myofibroblasts which take their place, prolonged and indefinite inflammation can results in cirrhosis or fibrosis of liver which is accompanied by permanent deterioration of liver functionality (Iwaisako *et al.*, 2012).

2.5.5 ER stress induced Apoptosis

One of the most marked apoptotic pathway which is activated as a result of ER stress is intermediated through CHOP as shown in figure 2.2 (Malhotra and Kaufman, 2007). CHOP(C/EBP homologous protein) or GADD153 (growth arrest and DNA damage 153) is one of the transcription factor (bZip) which is activated via the ATF6 and PERK signaling pathways under stress conditions (Ma *et al.*, 2002; Ron and Habener, 1992). Activation of CHOP causes the transcriptional expression of various genes which are involved in promotion of apoptotic cell death. Some of the above mentioned genes are *Gadd34*, *Ero1*, *Dr5* (death receptor 5), *Trb3* (Tribbles homolog 3), and carbonic anhydrase VI (Novoa *et al.*, 2001) . Permanent protein formation during the time when cell is under ER stress can generally results in activation of UPR and initiation of apoptotic signaling pathways (Marciniak *et al.*, 2004; Nagata *et al.*, 2004). It has been also reported that CHOP could be involved in the downregulation of expression of the antiapoptotic protein (BCL2), that results in increased oxidative stress and cause apoptosis (McCullough et al., 2001).

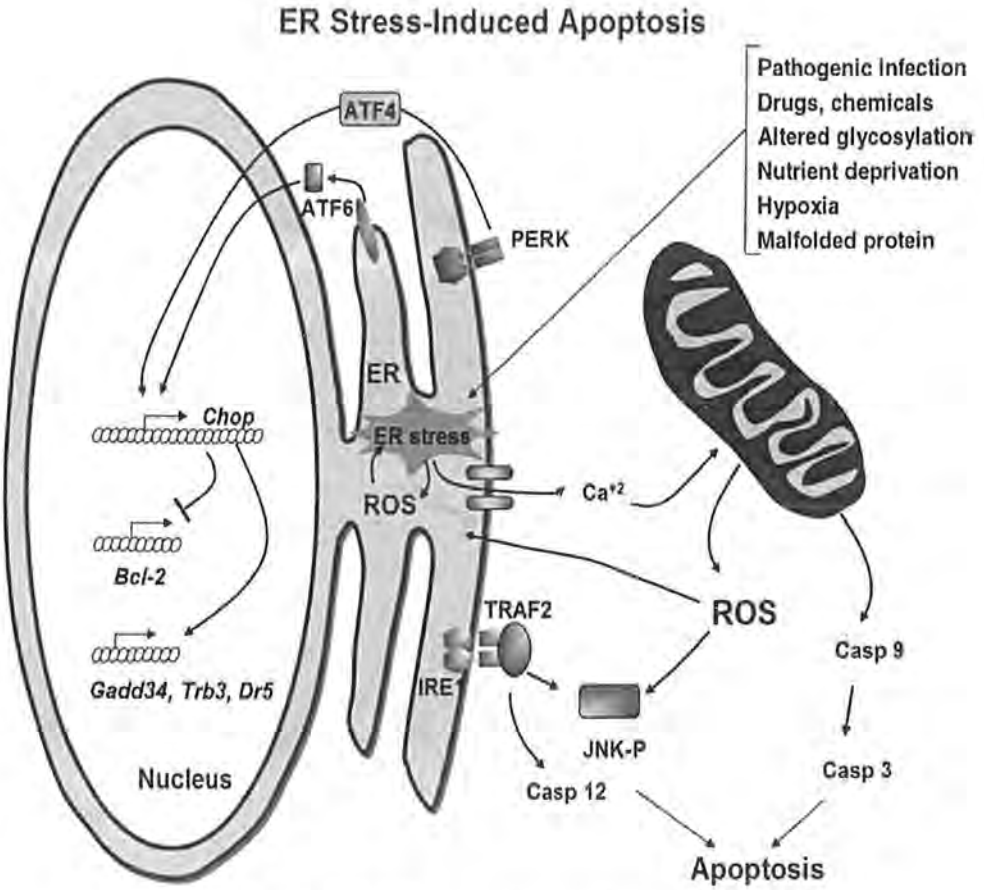

Figure 2.2 ER stress induced apoptosis

2.5.6. Histopathology of liver

Histopathology is the study of damaged tissues effected by any pathological condition under microscope. It is one of crucial diagnostic medical technique which is used for anatomical and histological studies of human and animal models microscopically. It is done by viewing a very thin tissue slice under light microscopes. This technique comprises of several different methods which are used to visualize the structural components of diseased tissue and cellular bodies microscopically and identify definite modifications in the structure in certain diseases (Paget and Thompson, 1979). Generally , tissues are immobilize with 10% formalin solution, fixed in paraffin, and then physically sliced by the help of microtome to several slices that are 4-5mm in thickness. After slicing paraffin wax is removed from these slices and dehydrated. Then they are subjected to staining with hematoxylin and eosin (H&E) or they can easily be utilized for other processes which includes distinct stains, immunohistochemistry,

fluorescence in situ hybridization (FISH) etc. Throughout the technique of histopathology, several phases and processes are delicate to guarantee typical and illustratable slices (Slaoui and Fiette, 2011).

Histology of liver is done by setting liver section in fixative and then embedded in paraffin for dense sustenance. 3-4 μm of thin slices are trimmed to stain with eosin and hematoxylin. These slides are then observed in light microscope. Carbon tetrachloride is a strong toxin which causes hepatotoxicity (Waring *et al.*, 2001). To evaluate the effect of carbon tetrachloride on biochemical and histopathological anomalies and protective function of amino acid taurine, an experiment was done on animal model (rat). Carbon tetrachloride treated group displays some sort of structural irregularities including ballooning, centrilobular necrosis and hepatocytes deterioration near central vein. Then slice six micron piece of liver tissue and stained it with eosin and hematoxylin to study histological changes in it. Several biomarkers such as total protein content and levels of some enzymes i.e. succinic dehydrogenase (SDH), alkaline phosphatase (Alp) and concentration of lipids were studied. Group with the treatment of CCl_4 depict disintegration of vacuoles, necrotic and apoptotic cell death of hepatocyte, fatty acid deterioration, and mononuclear cellular infiltration around the central vein. These modifications are determined in animals treated with carbon tetrachloride solely and the effect was more intense and acute than those animals which are given carbon tetrachloride with taurine (Hassan *et al.*, 2003).

Ethanol fraction of *Salix subserrata* display hepatoprotective potential when investigated in rat upon carbon tetrachloride exposure. Group with the treatment of carbon tetrachloride produced hepatic lesions together with extreme impairment of liver cells that were disturbed by the structure of portal vein and venous congestion (Wahid *et al.*, 2016).

Materials and Methods

Therapeutic and healing aptitude of plants is generally due to the presence of phytochemicals and other bioactive constituents, which are found in thousands of varieties of medicinal plants. Therapeutic activities of *L. pinnatifidum* are assessed by following in vitro and in vivo assays.

3.1. Plant collection

L. pinnatifidum whole plant was collected from Bagh, Azad Jammu Kashmir during April- June 2016. This plant was identified from Dr. Muhammad Zafar, Department of Plant sciences and provided with an accession number 175701 by Herbarium of Pakistan, Quaid-i-Azam University Islamabad.

3.2. Extract preparation

The whole plant was cleansed thoroughly to eradicate dust and other debris. For two weeks, plants were stored under shade, so that all the water content got vaporized and the plant dried wholly. In order to powder the plant material electric grinder was utilized, to get fine powder from it. For production of same sized particles, plant powder was passed through 60-mesh topology Willy Mill. Extraction of solvents was the next phase, in which powdered plant material was dipped, for some days, in pure methanol. To obtain refine methanolic plant extract, filtration was performed by using Whatman number.1 filter paper, that results in formation of filtrate which was then get dried via rotary vacuum evaporator, and then solvent was totally vaporized.

3.3. Fractionation

After methanolic extraction, fractionation was the next step. It was done in order to isolate compounds according to the ascending order of the polar nature from the crude extract. An amount of 50 g of LPM was mixed and dissolved in 200ml of distilled water. Then liquid-liquid partition was performed and fractions were collected by addition of solvents in below mentioned order, n-hexane (LPH), chloroform (LPC), ethyl acetate (LPE), n-butanol (LPB). The filtrate left behind after fractionation was also obtained and dehydrated to form aqueous fraction (LPA). For the evaluation of in vivo and in vitro biochemical analysis water content was removed from every fraction, they were weighed and stored at 4°C.

3.4. Phytochemical analysis

To detect the active phytochemicals found in *L. pinnatifidum* certain qualitative assays were done.

3.4.1. Qualitative assays

Different qualitative assays were done to detect certain biochemicals such as flavonoids, phenols, tannins, alkaloids, saponins, terpenoids, coumarins, anthocyanin and anthraquinones.

3.4.1.1. Assessment of phenols

Qualitative evaluation of phenols was done by following Harborne (1998) methodology. Each sample weighing 1mg was dissolved in 2ml of distilled water. After dissolution 10% of ferric chloride solution was added in it. Appearance of blue or green color indicates the presence of phenols.

3.4.1.2. Assessment of flavonoids

For flavonoids estimations Trease and Evans (1989) procedure was observed. Weigh 1mg of each sample and add it in separate test tubes. Then sodium hydroxide (2N) was dissolved in every test tube. Formation of yellow color symbolized the existence of flavonoids.

3.4.1.3. Assessment of coumarins

Methodology constructed by Harborne (1998) was followed. In 1 ml of every sample 10% of sodium hydroxide solution was added. Yellow color approves the presence of coumarins.

3.4.1.4. Assessment of saponins

Take 2 mg from each sample and then dissolve 2 ml of distilled Hydrogen peroxide in every test tube. After about 15 minutes the sample of reaction became thoroughly miscible. About 1-2 cm thick layer of soap consistency was formed which proved the presence of saponins (Harborne, 1998).

3.4.1.5. Assessment of tannins

Weigh 1mg of each sample and add it in separate test tubes then add 5% of ferric chloride solution . Formation of dark blue or greenish black color is the validation of tannins (Trease and Evans, 1989).

3.4.1.6. Assessment of terpenoids

Take 0.5 mg of each sample, in this first add 2 ml chloroform and then add sulphuric acid. Production of a red brown stained layer sandwiched in two layers specified the presence of terpenoids (Trease and Evans, 1989).

3.4.1.7. Assessment of anthraquinones

A quantity of about 1 mg from each sample was taken and dissolved in 2% of diluted hydrochloric acid. Creation of red color was a validation of the presence of anthraquinones (Harborne, 1998).

3.4.1.8. Assessment of anthocyanin and betacyanin

Weigh a quantity of 1 mg from each sample, then in these samples dissolve 2 ml of 1N sodium hydroxide. After addition of NaOH the reaction mixture was heated at 100 °C in water bath for approximately 10 minutes. Bluish green color was the confirmation of anthocyanin and yellowish color was the sign of betacyanin existence in the each sample (Trease and Evans, 1989).

3.4.1.9. Assessment of alkaloids

Add 2 ml of sulphuric acid in concentrated form and then add Mayer's reagent in them. From each sample take 2 mg concentrated sulphuric acid and was mixed in the test sample. Appearance of green color or formation of precipitation in white color represents the occurrence of alkaloids in the each sample (Trease and Evans, 1989).

3.5. *In vivo* studies

In order to study, the functional aptitude of hexane fraction of *L. pinnatifidum* by eliciting carbon tetrachloride (CCl_4) intoxication in rat liver. Defensive potential of plant under investigational study was detected in liver tissue. Tissues of liver were inspected at three levels i.e. biochemical, histological and molecular.

3.5.1. Study design

To perform this type of experiment, Shyu *et al.* (2008) protocol was observed. Sprague Dawley forty eight male rats of about 180-250 (g) weight were employed for this experiment. For the efficient conduction of experiment regulations of National Institute of Health were carefully followed. Then the formulated strategical plan was acknowledged by the Ethical Committee of Quaid-i-Azam University, Islamabad, Pakistan. With a 12 h dark/light time, rats were stored at standard temperature (25±3 °C) in ordinary cages. It was made sure that the animals were supplied with appropriate usual laboratory feed and clean water. Dose of the plant extract was given to the rats under study on alternative days for 1 whole month.

3.5.2. Drug formation

To mediate toxicity in rats, each group received calculated dose according to the rat's body weight.

3.5.3. Preparation of plant dose extract

In DMSO premeditated amount of hexane extract was mixed. Then its dilution was made with olive oil to acquire 10% of DMSO. Rats received this dose rendering to their body weight.

3.5.4. Grouping of rats

Rats were classified in seven different groups, for assessing the CCL_4 mediated hepatotoxicity and plant's potency in liver. Laboratory normal feed and clean water was accurately given to the rats under study. Supply of feed and water to the animal's cages was halted one day before initiating this experiment. Plant fraction and silymarin were presented orally via pigeon feeding tubes and CCl_4 was given through intraperitoneal shots. Below mentioned strategical plan illustrates groups and dose management.

Table3.1. Distribution of groups

Group number	Treatment
Group1 (Control)	No treatment
Group2 (CCl_4)	30%CCl_4 in 10% DMSO (1 mg/kg) i.p.
Group3 (CCl_4+Silymarin)	30%CCl_4+200 mg/kg of LPH
Group4 (CCl_4+LPH-low dose)	30%CCl_4+ 400 mg/kg of LPH
Group5 (CCl_4+LPH-high dose)	30%CCl_4+ 200 mg/kg of LPH
Group6 (LPH-low dose)	200 mg/kg
Group7 (LPH-high dose)	400 mg/kg

The next day before which experiment had ended successfully, rats were dissected and blood and samples of tissue were stored for additional assays. Dissection of rats was performed from ventral side of the animal body, blood was collected and liver was removed from the system. Next step was the centrifugation of blood sample which was obtained and serum was collected from the centrifuged blood and preserved at 4°C. Liver taken from the animal body was cleansed thoroughly in frozen saline solution and then treated with liquid nitrogen in order to store liver organ for enzymatic analysis and preserved at -20°C. Some part of liver was taken and stored in formalin solution for histopathological analysis.

3.6. Body weight and organ weight

Rats in all the groups were weighed at the start and then at the last day of experiment. Percentage increase of body weight was estimated by comparing initial and final body weight. During the time of dissection, liver was dissected and then placed in saline. After which relative weight of liver was calculated for each rat in various groups.

3.7. *In vivo* assays

To detect the defensive potential of hexane extract *L pinnatifidum* against CCl_4 mediated toxicity certain assays were done.

- Tissue analysis

- Serum analysis

3.7.1. Tissue analysis

In order to evaluate antioxidant enzymes and nitrite, protein, thiobarbituric acid reactive substances (TBARS), H_2O_2 and reduced glutathione (GSH) activities, assays were done for analysis of tissue.

3.7.2. Preparation of homogenate

Obtained tissues of liver was firstly weighed and 100 mg of every organ was collected and homogenized in 1 ml of K_2PO_4 buffer of 100 (mM) and EDTA having molarity 1 (mM) was mixed in the buffer and then pH was maintained at 7.4. Homogenate was centrifuged at 1500 *xg* at temp 4°C for half an hour. After centrifugation pallet was discarded and supernatant was obtained with which following enzymatic assays were done.

3.8. Biochemical analysis of tissue homogenate

With the help of several assays, antioxidant potency of plant extract was examined in liver tissues.

3.8.1. Protein estimation

Estimation of total protein in liver tissue, was examined by the help of procedure of Lowry *et al.* (1951). Weigh 80 mg of each liver sample and separate them for homogenization in phosphate buffer. Homogenate was centrifuged at 10,000 ×*g* rotary speed at 4℃ for 20 mins. Then about 0.1 ml of supernatant was exposed to incubation for 10 min, and was dissolved in 1 ml of alkaline solution. Then Folin Ciocalteu phenol reagent in similar proportion (1:1) was added in every eppendorf and then thoroughly vortexed it to dissolve. Incubate it at room temperature for half an hour and then absorbance was taken at wavelength 595 nm and total solubilized protein was estimated by bovine serum albumin standard curve formation.

3.8.2. Evaluation of antioxidant profile

Following assays were done for the evaluation of antioxidative nature.

3.8.2.1. Catalase assay (CAT)

With some alterations Chance and Maehly (1955) methodology was used to evaluate catalase activity. About 100 µl of supernatant of homogenate, 400 µl of H_2O_2 (5.9 mM) and 2500 µl of 50 (mM) of phosphate buffer (pH 5.0) were added and dissolved altogether. Alterations in absorbance of mixture was evaluated after 1 minute gap at 240 nm. Change in absorbance of 0.01 U/min is equals to 1U of CAT actvity.

3.8.2.2. Peroxidase assay (POD)

Activity of Peroxidase was investigated by utilizing Chance and Maehly (1995) protocol with few alterations. Reaction mixture for peroxidase assay comprised of 1000 µl of supernatant in 2500 µl of 50 (mM) phosphate buffer (pH 5.0), 300 µl of H_2O_2 (40 mM) and 100 (µl) of guaiacol (20 mM). After 1 minute gap transmission in optical density was evaluated at 470 nm. POD activity can be expressed as change in optical density of 0.01 U/min.

3.8.2.3. Superoxide dismutase, assay (SOD)

Superoxide dismutase activity estimation was done by using the methodology of Kakkar *et.al* (1984). Testing mixture of this assay comprised 0.1 ml of 186 (mM) phenazine methosulphate, 1.2 ml of 0.052 (mM) sodium pyrophosphate buffer and 0.3 ml of supernatant was mixed and subjected for centrifugation for 10 minutes at 1500 ×*g* and then at 10,000 ×*g* for 15 minutes. Reaction was start by introducing 0.2 ml of 780 (µM) NADH. At the end of reaction and after 1 minute glacial acetic acid was added. Absorbance was taken at 560 nm and the results are written in units/mg protein.

3.8.2.4. Reduced glutathione assay

With some adjustments in Jollow *et al.* (1974) methodology was utilized to calculate the reduced glutathione activity. Firstly 1000 µl of homogenate mixture was dissolved with 1000 µl (4%) of sulfosalicylic acid. Then this solution was stored at 4 °C for 60 mins then it is subjected to centrifugation at 4 °C for 20 minutes at 1200 ×*g*. collectively 3 ml of final master mixture has almost 2.7 ml phosphate buffer maintained at pH 7.4, 100 µl filtered solution and 200 µl of DTNB (100 mM). Intensity of colored solution was calculated by measuring O.D at 412 nm. Outputs of absorbance are recorded as µM GSH/g tissue.

3.8.2.5. Estimation of lipid peroxidation

Iqbal *et al.* (1996) protocol was followed for lipid peroxidation assessment. In this method, total 1000 µl master mixture was prepared which is composed of 580 µl of 0.1 (M) phosphate buffer of pH 7.4 , 200 µl of homogenate, 200 µl of 100 (mM) ascorbate and 20 µl of 100 (mM) ferric chloride solution. Reaction mixture was Incubated in quaking water bath for 60 mins at 37 °C temperature. Lastly 1000 µl of 10% trichloroacetic acid was mixed. Test tubes were put in boiling water bath, and addition of 1000 µl of 0.67% thiobarbituric acid took place at 20 minutes and then stored on the thawed ice-bath and then centrifugation of samples occur at 2500 ×*g* for 10 minutes. Pallet was discarded and supernatant was obtained and amount of TBARS was evaluated by taking absorbance in comparison to reagent blank at 535 nm spectometrically. Lipid peroxidation activity was recorded as nM TBARS/min/mg tissue at optimum temperature (37 °C) by applying molar extinction coefficient (1.56×10^5 M^{-1} cm^{-1}).

3.8.2.6. Hydrogen peroxide assay (H_2O_2)

By the outline of H_2O_2 –intermediated horseradish peroxidase supported oxidative reaction of phenol red, H_2O_2 assay was done (Pick and Keisari, 1981). H_2O_2 assay reaction consist of 2000 µl of tissue homogenate (dissolved in 1 ml of solution containing 0.28 (nM) of phenol red), 5.5 (nM) dextrose, 8.5 units of horse radish peroxidase and phosphate buffer (0.05 mM with pH 7.0) and this master mix was then allowed to incubate for 1 hour at optimum temperature (37 °C). After incubating lapse;10 µl NaOH (10N) was dissolved in the reaction samples to halt the reaction process. After this reaction mixture was centrifuged at 800 ×*g* for 5 min. Supernatant was obtained and OD was calculated at 610 nm in comparison to blank reagent. H_2O_2 amount was recorded as nm H_2O_2/min/mg tissue and oxidation of phenol red by H_2O_2 was taken as a standard curve.

3.8.2.7. Nitrite assay

For the validation of nitrite, nitrite assay Grisham *et al.* (1996) methodology was applied. Griess reagent was required for this assay. Master mix for this assay comprised of 1 ml of homogenate samples which were dissolved in 100 µl of 3.0 M NaOH and

5% $ZnSO_4$ solution. Then the samples were centrifuged for 15-20 min at 6400 × *g*. Supernatant was obtained and 20 µl was dissolved in with 1.0 ml Griess reagent. Optical intensity was collected and absorbance was taken at 540 nm. For defining the nitrite volume in tissue samples sodium nitrite curve was used.

3.9. Serum analysis

In order to perform Serum analysis, diagnostics kits of AMP Company was used.

3.9.1. Alkaline phosphatase estimation

For the evaluation of liver biomarker (ALP) was perform by applying the use of spectrophotometer and other reagents (Roche Diagnostics GmbH, Mannheim, Germany).

Principle

The basis of Alkaline phosphatase (ALP) test is hydrolysis reaction of 4-nitrophenylphosphate. As a consequence of this hydrolysis synthesis of 4-nitrophenol and inorganic phosphate took place. During the above mentioned reaction, inorganic phosphate was accepted by alkaline phosphate. A direct relationship was found between the production rate of 4-nitrophenol and ALP performance capability that is calculated at 405 nm wavelength.

$$\text{4-nitro phenol phosphate} + H_2O \longrightarrow \text{Phenol red} + \text{Phosphate}$$

Reagents

Two reagents are used for this assay. Reagent 1 consisted of 800 µl of DEA buffer (pH10.2) and magnesium chloride. Reagent 2 comprised of 200 µl of 4-nitrophenylphosphate and 200 µl of samples.

Working procedure

Working mixture was prepared by dissolution of above 2 reagents along with samples, they are thoroughly mixed to obtain homogenous mixture. This master mix was incubated for about 1 minute at 37 °C, optical density was evaluated at 405 nm. To validate the ALP potency in samples below mentioned formula was used.

$$\Delta A / \text{min} \times 3333 = \text{ALP activity (unit / litter) at 37 °C}$$

3.9.2. Alanine aminotransferase estimation (ALT)

Determination of ALT activity was carried out by the help of spectrophotometer along with reagents (Roche Diagnostics GmbH, Mannheim, Germany).

Principle

L-alanine is supposed to react with α-ketoglutarate in order to produce pyruvate and L-glutamate. Alanine aminotransferase is an enzyme which catalyze this reaction an is associated with the allocation of amino group while lactate dehydrogenase involved in the catalytic oxidation of NADH to NAD and reduction reaction of pyruvate. Absorbance was measured at 340 nm.

$$\alpha - \text{ketoglutarate} + \text{L} - \text{analine} \rightarrow \text{Glutamate} + \text{Pyruvate}$$

$$\text{Pyruvate} + \text{NADH} \rightarrow \text{Lactate} + \text{NAD} + H_2O$$

Reagents

Reagent 1 contains 800 µl of Tris-HCL, L-alanine, and Lactate dehydrogenase. Reagent 2 comprises of 200 µl of NADH, 2-oxoglutarate, Biocides and 50 µl of sample.

Working protocol

By intermixing of all reagents in an estimated amount, working solution was made and then in it sample was dissolved precisely. Then this master mixture was allowed to incubate for 120 seconds and O.D reading was recorded at 340 nm. Mean of three consecutive readings was utilized to evaluate the activity of ALT by applying following formula.

$$\Delta A / \text{min} \times 3333 = \text{ALT activity (unit / litter) at } 37\ ^\circ C$$

3.9.3. Aspartate aminotransferase evaluation (AST)

For AST calculation spectrophotometer alone with the use of reagents (Roche Diagnostics GmbH, Mannheim, Germany) occur.

Principle

Aspartate aminotransferase (AST) is an enzyme used to catalyze the reaction of L-Aspartate with 2-oxoglutarate by transferring amino group between them, as a result

transferring production of glutamate and oxaloacetate takes place. Also malate dehydrogenase is used to reduce oxaloacetate to malate in the presence of nicotinamide adenine dinucleotide (NADH).

$$\text{L- Aspartate} + \text{2- oxoglutarate} \rightarrow \textit{L-}\text{ Glutamate} + \text{Oxaloacetate}$$

$$\text{Oxaloacetate} + \text{NADH} + \text{H}^{+} \rightarrow \textit{L-}\text{ malate} + \textit{NAD}^{+}$$

Reagents

Reagent 1 consisted of 800 µl of Tris-HCL, L-aspartate, NADPH, MDH and also LDH. Reagent 2 consisted of 200 µl of α-ketoglutarate and 100 µl of sample.

Working protocol

Working solution was created by intermixing the above mentioned reagents and after this addition of sample occur. Then the reaction sample was incubated at an optimum temperature (37 °C). Mean of consecutive three readings was calculated at 340 nm with a gap of half a minute. AST activity was measured by using following formula.

$$\Delta A / \text{min} \times 3333 = \text{AST activity (unit / litter) at 37 °C}$$

3.9.4. Total protein estimation

Principle

Salt of copper dissolved in alkaline solution leads to the generation of complex which is colored, which estimated the total serum protein. Estimation of protein was done by using AMP diagnostics company kit also known as biuret method.

Working protocol

Take 10µl of sample or standard which is albumin in this case, was added in 1000 µl of reagent that was comprised of potassium sodium tartrate, $CuSO_4$, KI and NaOH. Blank for this assay was distilled water and not the sample itself. After this incubation of reaction mixture was done for 10 minutes at 37 °C, and O.D was observed at 550 nm.

Total protein estimation was evaluated by following formula.

$$(\text{O.D. of sample} / \text{O.D. of standard}) \times n$$

Where n is the standard concentration.

3.9.5. Albumin estimation

Estimation of albumin was carried out by Colorimetry technique, it was done by utilizing bromocresol green (BCG) by maintaining pH 4.20 via AMP diagnostics company kit.

Working protocol

Reagent comprised of succinate buffer, Brij 35 and bromocresol green. 1 ml of reagent was dissolved in 10 µl sample and standard in this case was bovine albumin. After this reaction solution was exposed to incubation for about 5 minutes, the O.D was calculated at 625 nm. Distilled H_2O_2 was employed as a blank for this assay. Concentration of serum albumin was estimated by the given formula.

$$\frac{\text{OD sample}}{(\text{OD standard})} \times n$$

Where n is standard concentration.

3.9.6. Globulin estimation

Values of globulin was estimated by applying (Total protein – albumin) formula.

3.10. Histopathological examination of tissues

For the estimation of histopathology paraffin fixed stained slices of liver was performed. To fix the biological samples and to prevent tissue decay and preserve their morphology a multi-step procedure was followed. For this purpose, fresh tissues of liver was cut into fine bit and preserved in 10% formalin. Further fixed tissues were washed in increasing concentration of alcohol i.e. 50%, 70%, 90% and 100%. The main purpose of this step was to support tissue on hard solid matrix and it made easier to cut the thin section of tissues. Last step was the preparation of slides by segmenting 3-4 µm thin layers of fixed tissue samples and then hematoxylin and eosin staining was carried out. With the help of light microscope (DIALUX 20 EB) slides were scrutinized at 40X and for photography HDCE-50B was used.

3.11. RNA extraction by Trizol Method

To study genes at the molecular level we extract RNA from the liver tissue sample of rat. Extraction of RNA was performed by Trizol method. For sanitization of RNA, Trizol reagent is used, and then it is imperiled to precipitation step which was done via ethanol (pure or 70%) along with a step which is comprised of washing RNA pellet so as to make sure that our pallet is free from any kind of contaminated guanidine thiocyanate (Meng and Feldman, 2010).

Working protocol:

1. Liver tissue was taken from the -40°C refrigerator and then about 100 mg of sample was weighed. Now take the tube and place it on crushed ice to avoid defrosting.

2. Take one of the sample from crushed ice and add 1 ml Trizol in it. It is not essential to thaw the tissue sample before addition of Trizol.

3. In next step Lysis of cells occur by using a P-1000 pipette, which is done by frequent pipetting to the point until whole the solution is homogenized. Incubation of the homogenized solution takes place at room temperature (25°C) for 5 minutes.

4. Now add 200 µl of chloroform, shake well with hands for about 15 times and incubate it at room temperature for 2 minutes.

5. Transfer the above homogenized solution to a 2-ml Eppendorf tube.

6. Centrifugation of mixture occur in the cold (4°C) for 15 min at 12,000Xg. Take the supernatant and transfer it into a new chilled 1.5-ml Eppendorf.

7. Add 200 µl of isopropanol which precipitates the RNA. Allow it to stand for 10 minutes at crushed ice. This is an ideal ending point.

8. Transfer the Eppendorf in a microcentrifuge at 4°C temperature. Place the lids facing towards the outside of centrifuge's rotor. Centrifuge it for 10 min at 12,000Xg. This time discard supernatant and take pellet. Now RNA should be easily seen at the bottom end of the Eppendorf on the side which was placed towards rotor's external side.

9. Pour the supernatant into a microcentrifuge Eppendorf. It is not essential to decant all of the present supernatant. Now 75% of ethanol is added and then Centrifuged for 5 minutes at 7,500Xg.

10. Again pour the supernatant into the same microcentrifuge Eppendorf utilized for the isopropanol precipitation step. Short spin the Eppendorf having the RNA for few seconds to transport the remaining ethanol to the bottom end of the tube.

11. With the help of a pipette, decant much of the remaining ethanol, by taking extra care not to touch the RNA pellet. Addition of 1ml of extra pure ethanol (100%) occur and then centrifuge it again for 5 minutes at 7500Xg .

12. Dissolve the RNA pellet in 30–50 ml of RNAse free distilled water of molecular grade and take the tubes on crushed ice. RNA can then be preserved at -80°C.

3.11.1. Quantitative and qualitative analysis of RNA

After the successful isolation of RNA is now subjected for quantitative and qualitative analysis. For quantitative analysis NanoDrop is performed while for the qualitative analysis 1% Agarose gel electrophoresis was done.

3.11.2. Gel electrophoresis of extracted RNA

Gel electrophoresis was performed to investigate the integrity of total RNA extracted from tissue sample using normal non-denaturing Agarose gel medium.

3.12. cDNA synthesis

cDNA was synthesized from extracted RNA for RT PCR analysis. cDNA synthesis was done by using thermo fisher scientific Revert Aid First Strand cDNA Synthesis Kit. Figure 2 explained the procedure of cDNA synthesis (Meis and Khanna, 2009).

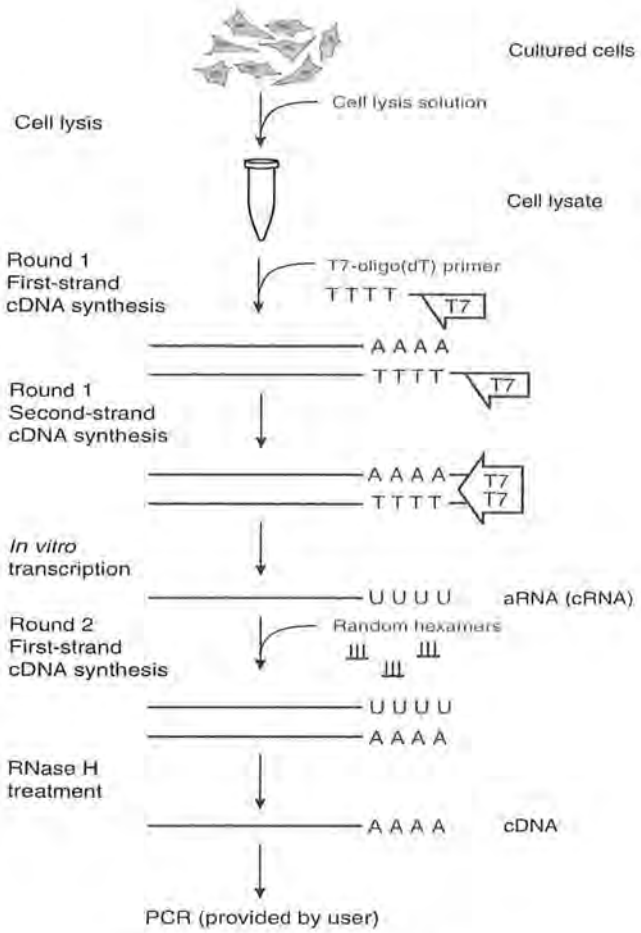

Fig. 3.1. cDNA synthesis

Working protocol

- Total extracted mRNA was transformed into cDNA by the help of thermo scientific revert aid first strand cDNA synthesis kit.
- Thaw all the constituents of the kit and then they were briefly spin at 2500 rpm for half a minute and then kept on crushed ice.
- Components of table 3.2 were placed in an autoclaved tube and placed on ice.

Table 3.2. Ingredients of cDNA synthesis

Ingredients	**Per reaction (µl)**
Template RNA	**12**
DEPC water	**0.3**
Oligo (dT) 18 primer	**0.2**
Buffer	**4**
Ribo Lock RNASE Inhibitor	**0.5**
10Mm DNTP Mix	**2**
Reverse Transcriptase	**1**
Total Volume	**20**

- All constituents was intermixed slightly and centrifuged for 30 seconds.
- Oligo (dT) 18 was incubated for 1 hour at 42°C temperature.
- The reaction was ceased after the provision of heat to the master mix at -70°C for 5 minutes. The output of this reaction might be preserved at -20°C for not more than a week or could be utilized to produce amplified product of PCR of a specific gene. If one wanted to preserve cDNA for longer period of time, it should be stored at -70°C.

3.13. RT PCR

RT-PCR (Real Time Polymerase Chain Reaction) is widely used to magnify cDNA products from the mRNA that are reverse transcribed (Pfaffl, 2001). RT PCR was performed to check the expression of the target genes affected by CCL_4 administration. For establishing the relative quantification Beta Actin was taken as a control or reference gene to which expression level of target genes would be compared. Primers were designed for all genes under study and commercially synthesized from BIG, Hong Kong. All the sequences of primers have been given in Table 3.3.

Table 3.3. Primer sequence of genes

S.#	Primer Name	Sequence
1.	GRP78-F	5′-GAAATTTCTGCTATGGTTCTCACT3′
2.	GRP78-R	5′-GAAGTAAGCTGGTACAGTCACA-3′
3.	GCLC-F	5′-GTGGACACCCGATGCAGTAT-3′
4.	GCLC-R	5′-TCATCCACCTGGCAACAGTC-3′
5.	XBPS-F	5′-TGAGTCCGCAGCAGGTGCA-3′
6.	XBPS -R	5′-ACAGGGTCCAACTTGTCCAGAA-3′
7.	XBPT-F	5′-CCCTGGTTACTGAAGAGGTC-3′
8.	XBPT-R	5′-GTCCAACTTGTCCAGAATGC-3′
9.	XBPU-F	5′-AAAGCGCTGCGGAGGAAA-3′
10.	XBPU-R	5′-AGCTGGAGTTTCTGGTTCTCT-3′
11.	TNF-α-F	5′-GCTCCCTCTCATCAGTTCCA-3′
12.	TNF-α-R	5′-GGTTGTCTTTGAGATCCATGC-3′
13.	IL-6-F	5′-GTCAACTCCATCTGCCCTTC-3′
14.	IL-6-R	5′-ACTGGTCTGTTGTGGGTGGT-3′
15.	MCP-1-F	5′-CAAGATGTGCGCTGAGGACA-3′

16.	MCP-1-R	5′-TTCTCTATTGGTGGCAGACC-3′
17.	CHOP-F	5′-CCTGTCCTCAGATGATGAAATTG-3′
18.	CHOP-R	5′-CTAGGGATGCAGGGTCAAGA-3′
19.	BCL-2-F	5′-TGGATGACTGAGTACCTGAACC -3′
20.	BCL-2-R	5′- CAGCCAGGAGAAATCAAACAG -3′
21.	Col 1-α-F	5′-GTCCCCGAGAGGAAACAATG-3′
22.	Col-1-α-R	5'-ACCACGCATTCCCTGAAGA-3′
23.	B-Actin-F	5'-CCTCTATGCCAACCATGC-3'
24.	B-Actin-R	5'-CATCGTACTCCTGCTTGCTG-3'

Working protocol

Firstly, conditions for RT-PCR (Mygo Pro, Model= EAL1701F-240) were optimized. By using these optimized conditions RT-PCR was carried out for B-Actin, GRP78, GCLC, XBPS, XBPT, XBPU, TNF-α, IL-6, MCP-1, CHOP, BCL-2 and Col-1-α. Real Time PCR conditions are given in the table 3.4. RT-PCR was done with the help of machine of RT Mygo, Ireland. RT-PCR reactions mixture were collected by making a master mixture which consisted of primers of genes under study, SYBR Green dye and RNAse free water. 10µl of reaction mixture was made by the addition of below mentioned components in definite order into RT-PCR tubes (Table 3.5). The results of expression levels were also established by agarose gel electrophoresis by utilizing products of RT-PCR.

3.13.1. Relative quantification of gene expression

Relative quantification of several genes was evaluated by comparative CT method. β-actin gene was used as internal or reference gene. For this purpose ΔCT of treatment based (CT of treated - CT of internal control) and ΔCT of control sample (CT of control

- CT of internal control) for target gene was estimated. Then ΔΔCT = ΔCT (treated) - ΔCT (control) was evaluated. For calculation of fold change because of treatment=$2^{-\Delta\Delta CT}$ was followed (Schmittgen *et al.*, 2000). Probability values less than 0.05 were known to be significant.

Table 3.4 Conditions for qRT-PCR

Programs name		**Temperature °C**	**Ramp (°C/s)**	**Hold (s)**
Hold		95	4	600
3-step amplification	**Denaturation**	95	5	15
	Annealing	60	4	30
	extension	72	5	30
Pre-melt hold		95	5	10
High resolution Melting	**Initial stage**	60	4	60
	Final stage	97	0.05	15

Table 3.5: RT-PCR reaction volume with SYBR Green

i.	2x SYBR Green	5µl
ii.	10µM Forward Primer	0.25µl
iii.	10 µM Reverse Primer	0.25µl
iv.	Genomic DNA	0.5µl
v.	PCR water	4µl

3.14. Statistical analysis

For the determination of significances among several in vivo studies, one-way ANOVA was performed by using statistics 8.1 software. In order to determine comparison between different investigational groups for fold change values of expressional levels of genes Graph pad prism, one-way ANOVA and Tukey's tests were utilized. Cap software was utilized for image examination. The results were recorded as mean ± standard deviation which is mean ± SD. Level of significance was also observed and a p-value of ≤ 0.01 was assumed to be considered as significant in all assays and studies performed. For the analysis of serum recording Graph pad prism 5software was utilized. The results of experiments were recorded as mean ± standard deviation such as mean ± SD. By one way ANOVA ** $p < 0.01$.

Results

4.1. Yield of fractions of *Lepidium pinnatifidum*

The 50 g yield was collected from 1.4 kg powdered whole plant of the *L. pinnatifidum* by using commercial methanol extraction procedure. Total amount of 40 g of methanolic extract, named as LPM, was fractionated by using liquid-liquid partition technique which is performed on its several polar and non-polar fractions. The amount of hexane fraction (LPH) was 6.1 g, 3.3 g chloroform (LPC), 2.2 g ethyl acetate (LPE), 5.5 g butanol (LPB) and 19 g of aqueous fraction (LPA) as demonstrated in Figure 4.1. several solvents show following order during fractionation: LPA > LPH > LPB > LPC > LPE.

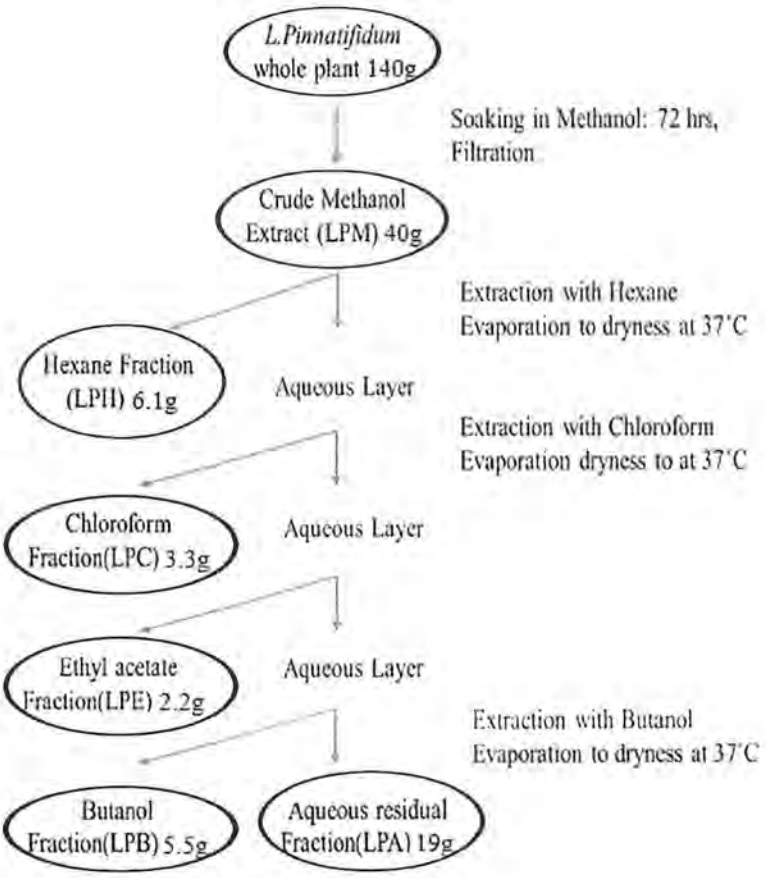

Figure 4.1: Flow sheet of fractionation of *L. pinnatifidum*

4.2. Phytochemical analysis

4.2.1. Qualitative examination of *L. pinnatifidum* fractions

In order to detect several kinds of phytochemicals present in *L. pinnatifidum* hexane fraction and its other extracts qualitative assays were carried out. Results of which are illustrated in the (Table 4.1.) These results ensure the incidence of alkaloids and tannins in every fraction of *L. pinnatifidum* . LPH fraction did not contain any cumarins while all other fractions did have them. Phenolic and flavonoid content present in all fractions except LPC. LPM fraction contains anthocyanins while betacyanins was found in other fractions. Except LPM and LPA all fractions have saponins. LPH and LPC have terpenoids whereas anthraquinones were not present in any fraction of *L. pinnatifidum.*

Table 4.1: Qualitative analysis of *L. pinnatifidum*

Phytocheimcals	LPE	LPA	LPM	LPC	LPB	LPH
Terpenoids	-	-	-	-	-	++
Cumarins	+++	+	+++	+	++	-
Flavonoids	+++	++	+++	_	++	++
Tannins	+++	++	+++	++	++	++
Anthraquinone	-	-	-	-	-	-
Phenols	+++	++	+++	-	++	+
Alkaloids	+	++	++	+	+	+
Saponins	++	-	-	+	+++	++
Anthocyanin &	+++*	+*	+**	+*	++*	-
Betacyanin						

(+) component present, (++) moderate concentration of component, (+++) highest concentration of component, (-) component absent, (**) anthocyanin, (*) betacyanin

4.3. *In vivo* protective potency of LPH

CCl_4 is one of the deadly biotoxin generally known as hepatotoxin and can be the cause of severe oxidative stress, hepatic toxicity and also affect several organs in animal models. CCl_4 hepatotoxicity occur because of the generation of many types of free radicals which can cause tissue damage in the body. These free radicals have the ability to bind with certain macromolecules including nucleic acids, proteins and lipid molecules. Lipid peroxidation is one of the deleterious effect of CCl_4 toxicity which activates macrophages and phagocytic cells leading to the activation of apoptotic and necrotic pathways as well as several inflammatory signaling pathways to retrieve the damage caused by liver toxicity. The *in vivo* antioxidant experiments show that *L. pinnatifidum* hexane (LPH) extract have potential to defend the body from the oxidative damage, due to these results we perform *in vivo* studies to access the antioxidant potential of L. *pinnatifidum* hexane extract on animal models. CAT (catalase), GSHPx (glutathione peroxidase) and SOD (superoxide dismutase) are classified as enzymatic antioxidants. CAT and GSH can convert lipid hydroperoxides to less toxic alcohols and also detoxify hydrogen peroxide that is generated during SOD catalyzed super anion dismutation.

4.4. Protective aptitude of LPH on CCl_4 mediated hepatotoxicity

The potential of *L. pinnatifidum* hexane extract to provide protection against CCl_4 induced toxicity in liver tissue was assessed by calculating following parameters.

4.4.1. Protective effects of LPH on body weight and organ weight

Table 4.2 show the effectiveness of LPH towards CCl_4 mediated toxicity on percentage increase in body weight, absolute and relative liver weight. Administrating CCl_4 markedly ($p < 0.05$) repressed the percent increase in normal weight gain in the body mass whereas increase absolute and relative weight of liver in rodents with comparison to control ones. Co-administration of *L. pinnatifidum* hexane fraction retreated the obnoxious results of CCl_4 and enhanced the percent gain in weight of the body while a significant repression in absolute and relative liver weight was exhibited in the experimental groups. Co-administration of silymarin along with CCl_4 to rats of all groups except control retreated the body as well as organ weights close to control group.

Moreover, no significant ($p > 0.05$) difference was observed after the treatment of low (200 mg/kg bw) and high (400 mg/kg bw) dose of LPH alone.

4.4.2. Protective role of LPH on liver serum enzymes

To monitor the general functions of liver specific biomarkers are assessed, it contains modifications in tissue and cellular integrity, which is detected by the release of specific cell constituents including alanine aminotransferase (ALT), alkaline phosphatase (ALP) and aspartate aminotransferase (AST) into serum. High levels of AST and ALT in serum show hepatocytic damage and elevated levels of ALP indicates hepatobiliary injury. Oxidative stress produced as a result of CCl_4 exposure leads to deterioration of liver tissue and affect liver bio markers normal activities. CCl_4 induces hepatocellular injury and to assess this injury and cure via plant , we determine level of these biomarkers in serum. Normal and modified functioning of liver biomarkers in different treatment groups are described in Table 4.3 along with bar graph (figure 4.2.4.3,4.4). There is marked elevation ($p<0.05$) in serum biomarker levels in CCl_4 treated group. This hepatotoxicity is stabilized by the co-administration of LPH doses. By the treatment with LPH in dose dependent manner, high levels of serum enzymes are markedly restored towards normal.

4.4.3. Protective role of LPH on serum protein profile

Another parameter to detect liver disorders is the increase levels of protein, albumin and globulin. Serum proteins are known to show antioxidant potency because of the presence of relatively high amount of free thiol groups. Among all those proteins, albumin is one of the most effective and abundant antioxidant present in extracellular matrix. The function of albumin as one of the member of serum antioxidant defense system, is notably prominent to be used as an authentic and dependable marker of oxidative stress. Globulins together with albumin are the two most important components of serum total proteins. The globulins play a significant function in inflammation and immunity. Albumin is among the proteins that are produced chiefly by the liver and in severe liver injuries its level get decreased. All of these proteins are measured in mg/dl. It is detected that with comparison to control group, levels of total

proteins decreases in CCl_4 treated group (Table 4.4).Total protein levels in serum are markedly ($p<0.05$) restored by the LPH dosage in a dose dependent manner.

4.4.4. Protective role of LPH on antioxidant enzymes

In order to protect a living organism from the deleterious effects of oxidative stress, living body has an innate defense system. CAT, POD and SOD are antioxidants that are naturally present in the body, while dietary antioxidants are also provided to body exogenously. CAT is used as a catalyst in decomposition reaction that converts hydrogen peroxide to H_2O and O_2. It is one of the crucial enzyme that provides protection to the cellular integrity against oxidative stress. CAT contains the topmost turnover numbers amongst all the enzymes, one molecule of CAT can change millions of H_2O_2 molecules into H_2O and O_2 in one second. Superoxide dismutase (SOD) acts as a catalyst in dis-mutation reaction of superoxide radical and converts it into either oxygen (O_2) molecule or hydrogen peroxide (H_2O_2). Peroxidases are the enzymes that are used to catalyze the reduction reaction of H_2O_2, organic hydroperoxides, and peroxynitrite. LPH has protective aptitude which was evaluated by evaluating the transformed level of antioxidant enzymes by providing CCl_4 and LPH in dose dependent manner. The results are illustrated in table 4.5 and figure 4.5,4.6,4.7) and our observation show that CAT,POD and SOD levels are significantly reduced in CCl_4 treated group. LPH high (400 mg/kg) and low dose (200 mg/kg) were given to restore the normal activities of all these enzymes. The LPH in higher concentration is more effective as compared to low dose of LPH.

4.4.5. Protective role of LPH on protein and GSH content

Protein and GSH levels in liver tissues are assessed and estimated in table 4.6 and figure 4.8). In CCl_4 treated group GSH and protein levels were deliberately decreased with comparison to control group. LPH and CCl_4 when co-administered, treated the CCl_4 toxicity in a dose dependent treatment. LPH (400 mg/kg) markedly retreat the CCl_4 induced toxicity as compared to control group.

4.4.6. Protective role of LPH on H_2O_2, nitrite and TBARS

The strength of CCl_4 induced hepatotoxicity was established by calculating the levels of nitrite, H_2O_2 and TBARS. Nitrite, H_2O_2 and TBARS levels were increased upon treatment with CCl_4 as illustrated in table 4.7, while LPH dose markedly retreat the CCl_4 toxicity and normalize the levels of these enzymes in a dose dependent manner. Moreover, LPH alone treated groups did not illustrate any marked change in the concentrations of TBARS, nitrite and H_2O_2 as compared to control group.

4.4.7. Protective role of LPH on histology of liver

The histopathological calculation revealed a significant method to examine and detect the biochemical activities .Eosins and hematoxylin (H&E) are the two major types of stain which are utilized to stain minute, slender pieces of hepatic tissue which are used to observe under microscope and photographed in magnified form i.e.10X and 40X. Control group have shown unchanged structural integrity like kupffer cells, characteristic central vein, sinusoidal and hepatocytes in figure 4.22 (A)change. As the liver was exposed to 30% CCl_4 (1 ml/kg b.w) on alternative days to induce hepatotoxicity by producing apoptosis, cellular hypertrophy, accumulation of inflammatory cells and dilation of central vein, as illustrated in figure 4.22 (B) Silymarin (200 mg/kg b.w) which is a standard drug, was given to restore structural abnormalities and cellular anomalies as shown in figure 4.22 (C). *L. pinnatifidum* hexane fraction (LPH) was given to reduce the hepatic damage by retreating the blood vessel dilations and hepatocytes morphology. High dose of LPH (400 mg/kg) showed highest hepatoprotective aptitude by reducing the deleterious effects and by restoring the transformed morphology to the normal figure 4.22 (E). By the provision of low dose of LPH (200 mg/kg), there still were few signs of CCl_4 toxicity like disruption of central vein 4.22 (D). LPH (High dose 400 mg/kg and low dose 200 mg/kg) alone treated group did not display any fatal effects on histology figure 4.22 (F,G). Histology of liver has proved the hepatoprotective aptitude of *L. pinnatifidum*.

4.4.8. Hepatoprotective potential of LPH on ER stress markers, inflammatory mediators and anti-apoptotic markers

The mRNA expression levels of ER stress genes are shown in (Fig. 4.9,4.10,4.11,4.12) The expression of GRP78, XBP-1 s, XBP-1 t and XBP-1 u was markedly ($p < 0.05$) upregulated in the case of CCl_4 treated group as compared to control group. Co-administration of silymarin regularized the fold change in comparison to CCl_4 mediated stress. Groups treated with LPH low (200 mg/ kg bw) and high dose (400 mg/kg bw) along with CCl_4 significantly ($p < 0.05$) repressed the expression level of all stress inducing genes mentioned above in comparison to that of CCl_4 treated group. But, treatment with LPH extract alone revealed non-significant ($p > 0.05$) restored the fold change of these genes markedly.

Administration of CCl_4 to animal model significantly ($p < 0.05$) increased the expression level of antioxidant enzyme GCLC to that of the control ones. Co-administration of silymarin and LH at both low and high doses reduced the expression level of this particular gene to that of CCl_4 treated group. However, there was no significant ($p > 0.05$) change in fold change of GCLC in group treated with CCl_4, silymarin and CCl_4 with high dose of LPH (400 mg/kg bw) group significantly. (Fig. 4.13)

The expression level of pro-inflammatory cytokines (IL-6, TNF-α) and chemokine (MCP-1) was markedly ($p < 0.05$) enhanced in group receiving treatment with CCl_4 alone. Administration of at low dose (200 mg/kg bw) and high dose (400 mg/kg bw) of LPH together with CCl_4 also revealed significant increase in the expression level of above mentioned genes. However, administrating LPH alone normalize the expression close to the control group. (Fig. 4.14,4.15,4.16)

Expression of anti-apoptotic marker gene Bcl-2 was significantly ($p < 0.05$) reduced in the rats treated with CCL_4 alone. Co-administration of Silymarin with CCL_4 regulated the expression to near to the control group. Also, there was no marked difference revealed in fold change of this gene in groups receiving only the treatment of LPH at high (400 mg/kg bw) and low (200 mg/kg bw) dose.(Fig. 4.17). Expression of apoptotic gene CHOP was significantly ($p < 0.05$) elevated in the group treating with CCL_4 alone. Co-administration of Silymarin along with CCL_4 regularized the

expression close to the control expression level. However, there was no significant difference found in fold change in rats getting treatment with LPH both low (200 mg/kg bw) and high dose (400 mg/kg bw) of LPH. (Fig. 4.18)

Expression of the gene COL1A was also significantly ($p < 0.05$) elevated in the rats with CCL_4 treatment. Co-administration of CCL_4 with Silymarin showed reduction of expression near to that of control group while administration of high dose (400 mg/kg bw) of LPH showed no difference in fold change. (Fig 4.19)

The gel images obtained from electrophoresis of these genes have been illustrated in Fig. 4.20. This gel revealed the respective regularization of genes as demonstrated by the RT-PCR assessment.

Table 4.2: Effect of *L. pinnatifidum* on body weight and organ weight of rats

Treatment group	**Initial body weight (g)**	**Final body weight (g)**	**% increase in body weight**	**Absolute liver weight (g)**	**Relative liver weight**
Control	122±1.00	182±1.8	51.7 ± 0.3^{a}	6.2 ± 0.03^{f}	$34.3\ 0.4^{e}$
CCl_4(1 mg/kg)	122± 1.5	160± 1.8	32.3 ± 0.5^{g}	8.7 ± 0.11^{a}	54.8 ± 0.8^{a}
CCl_4+Sily (200 mg/kg)	123± 2.3	179± 3.3	49.1 ± 0.4^{c}	6.1 ± 0.04^{e}	34.1 ± 0.6^{e}
CCl_4+LPH(400mg/kg)	121± 2.1	166± 2.5	39.3 ± 0.1^{e}	8.0 ± 0.07^{c}	45.5 ± 0.7^{c}
CCl_4+LPH(200 mg/kg)	123± 2	163± 2.5	34.3 ± 0.2^{f}	7.5 ± 0.1^{b}	49.6 ± 0.9^{b}
LPH (400 mg/kg)	122± 1.2	185± 2.7	50.6 ± 0.4^{b}	6.8 ± 0.1^{d}	36.8 ± 0.9^{d}
LPH (200 mg/kg)	124± 2.0	182± 2.6	47.5 ± 0.4^{d}	6.8 ± 0.08^{d}	37.6 ± 0.8^{d}

Mean ± SD (n=7), Means with different superscript letters specify significance at $p<0.05$, LPH; *Lepidium pinnatifidum* hexane extract, CCl4; Carbon tetrachloride, Sily; Silymarin.

Table 4.3: Effect of *L. pinnatifidum* on serum enzymes

Treatment	**ALT (U/l)**	**AST (U/I)**	**ALP (U/I)**
Control	65.5 ± 1.2^{h}	44.9 ± 1.9^{h}	54.8 ± 2.6^{h}
CCl_4 (1 mg/kg)	134.8 ± 1.1^{a}	114.5 ± 1.8^{a}	127.2 ± 2.1^{a}
CCl_4+Sily (200 mg/kg)	94.3 ± 1.0^{e}	75.2 ± 2.5^{e}	85 ± 2.4^{e}
CCl_4+LPH (400 mg/kg)	114 ± 2.1^{c}	95.9 ± 1.9^{c}	105.2 ± 1.9^{c}
CCl_4+LPH (200 mg/kg)	124.8 ± 1.5^{b}	105 ± 2.2^{b}	115 ± 2.0^{b}
LPH (400 mg/kg)	88.5 ± 1.1^{f}	64.6 ± 1.7^{f}	76.3 ± 2.9^{f}
LPH (200 mg/kg)	102.5 ± 2.1^{d}	85.6 ± 2.2^{d}	94.7 ± 2.5^{d}

Mean ± SD (n=7), Means with different superscript letters specify significance at $p<0.05$, LPH; *Lepidium pinnatifidum* hexane extract, CCl_4; Carbon tetrachloride, Sily; Silymarin

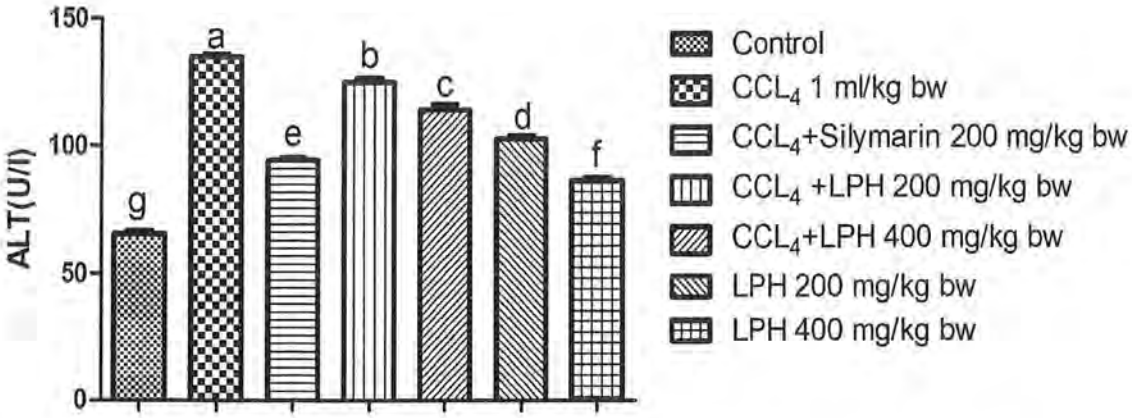

Figure 4.2: Effect of different treatments of *L. Pinnatifidum* on liver serum marker; ALT (Alanine aminotransferase), LPH *L. Pinnatifidum* hexane fraction, CCL_4 Carbon tetrachloride

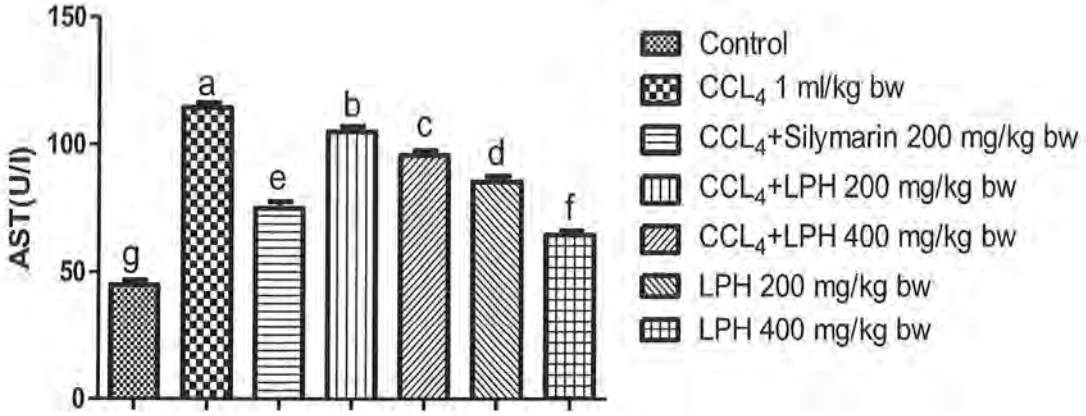

Figure 4.3: Effect of different treatments of *L. Pinnatifidum* on AST (Aspartate aminotransferase)

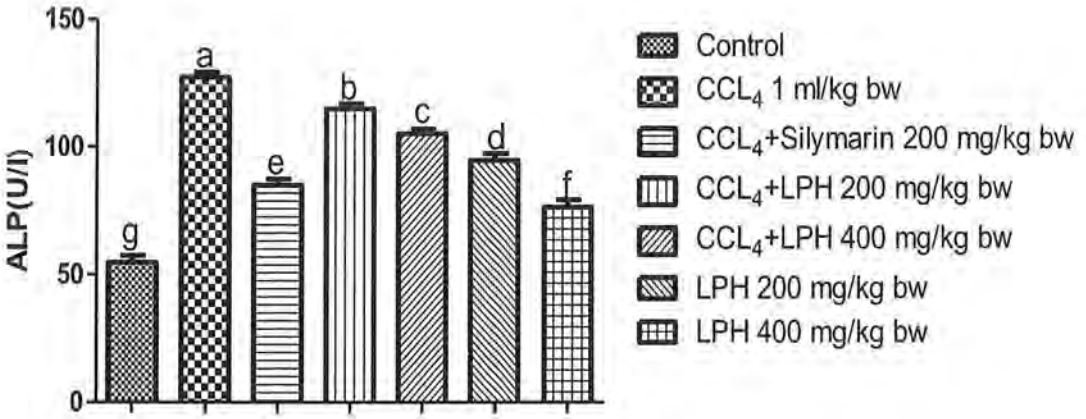

Figure 4.4: Effect of different treatments of *L. Pinnatifidum* on ALP (Alanine phophatase)

Table 4.4: Effect of *L. Pinnatifidum* on liver serum proteins

Treatment	Serum protein (mg/dl)	Albumin (mg/dl)	Globulin (mg/dl)
Control	103 ± 0.62^{a}	62.2 ± 0.29^{a}	33.2 ± 0.3^{a}
CCl_4+Sily (200 mg/kg)	87.1 ± 0.35^{d}	46.2 ± 0.38^{d}	22.4 ± 0.27^{d}
CCl_4+LPH (400 mg/kg)	77.1 ± 0.25^{f}	36.3 ± 0.38^{f}	17.4 ± 0.35^{f}
CCl_4+LPH (200 mg/kg)	70.5 ± 0.38^{g}	31 ± 0.32^{g}	13.9 ± 0.42^{g}
LPH (400/kg)	96.1 ± 0.32^{c}	51.3 ± 0.40^{c}	25.2 ± 0.14^{c}
LPH (200/kg)	83.1 ± 0.40^{e}	41.7 ± 0.51^{e}	19.2 ± 0.58^{e}
CCL_4 (1mg/kg)	66.2 ± 0.38^{h}	23.1 ± 0.50^{h}	11.1 ± 0.29^{h}

Mean ± SD (n=7), Means with different superscript letters specify significance at $p<0.05$, LPH; *L. pinnatifidum* hexane fraction, CCl_4; Carbon tetrachloride, Sily; Silymarin

Table 4.5: Effect of *L. pinnatifidum* on liver antioxidant enzymes

Treatment	CAT (U/min)	SOD (U/min)	POD (U/min)
Control	17.3 ± 0.44^{a}	7.2 ± 0.45^{a}	3.3 ± 0.31^{a}
CCl_4 (1ml/kg)	10.1 ± 0.18^{f}	1.1 ± 0.22^{e}	1.3 ± 0.32^{d}
CCl_4+Silymarin (200mg/kg)	12.4 ± 0.28^{b}	6.7 ± 0.47^{ab}	2.9 ± 0.4^{ab}
CCl_4+ LPH (400 mg/kg)	11.4 ± 0.37^{d}	4.3 ± 0.05^{d}	1.9 ± 0.22^{cd}
CCL_4+ LPH (200 mg/kg)	10.7 ± 0.24^{e}	3.9 ± 0.04^{d}	1.6 ± 0.51^{d}
LPH (400 mg/kg)	14.5 ± 0.36^{c}	6.4 ± 0.41^{b}	3.1 ± 0.58^{ab}
LPH (200 mg/kg)	11.6 ± 0.38^{d}	5.5 ± 0.11^{c}	2.5 ± 0.47^{bc}

Mean ± SD (n=7), a-e (Means with different letters) specify significance at $p<0.05$, LPH; *L. pinnatifidum* hexane fraction, CCl_4; Carbon tetrachloride

Table 4.6: Effect of *L. pinnatifidum* on liver proteins level and GSH

Treatment	**Protein tissue (µg/mg)**	**GSH (µM/mg)**
Control	11.6±0.07[a]	21.8±0.2[a]
CCl_4 (1ml/kg)	8.12±0.02[g]	3.4±2.64[e]
CCl_4+ Silymarin (200 mg/kg)	10.74±0.03[b]	21.7±0.6[b]
CCl_4+LPH (400 mg/kg)	8.5±0.02[e]	15.64±1.31[d]
CCl_4+LPH (200 mg/kg)	8.2±0.02[f]	15±0.15[d]
LPH (400 mg/kg)	10.5±0.09[c]	19.7±0.11[b]
LPH (200 mg/kg)	10.2±0.03[d]	18.8±0.19[b]

Mean ± SD (n=7), a-e (Means with different letters) specify significance at $p<0.05$, LPH; *L. pinnatifidum* hexane extract, CCl_4; Carbon tetrachloride

Table 4.7: Effect of *L. pinnatifidum* on liver proteins TBARS, H_2O_2 and Nitrite

Treatment	TBARS	H_2O_2	Nitrite
Control	53.8±0.5[g]	6.0±0.11[g]	61.6±4.0[f]
CCl_4 (1 ml/kg)	104±3.2[a]	18.8±0.76[a]	123.9±2.8[a]
CCl_4 + Silymarin (200 mg/kg)	78.0±1.0[e]	9.1±0.22[e]	81.5±1.99[d]
CCl_4 + LPH (400 mg/kg)	89.4±2.1[c]	14.8±0.92[c]	93.9±1.0.4[c]
CCl_4 + LPH (200 mg/kg)	97.9±1.14[b]	17.4±0.4[b]	104.9±3.02[b]
LPH (400 mg/kg)	71.6±1.0[f]	7.9±0.29[f]	71.7±2.20[e]
LPH (200 mg/kg)	84.3±1.43[d]	11.1±0.44[d]	89.4±1.50[c]

Mean ± SD (n=7), a-f (Means with different letters) specify significance at $p<0.05$, LPH; *L. pinnatifidum* hexane fraction, CCl_4; Carbon tetrachloride, Sily; Silymarin

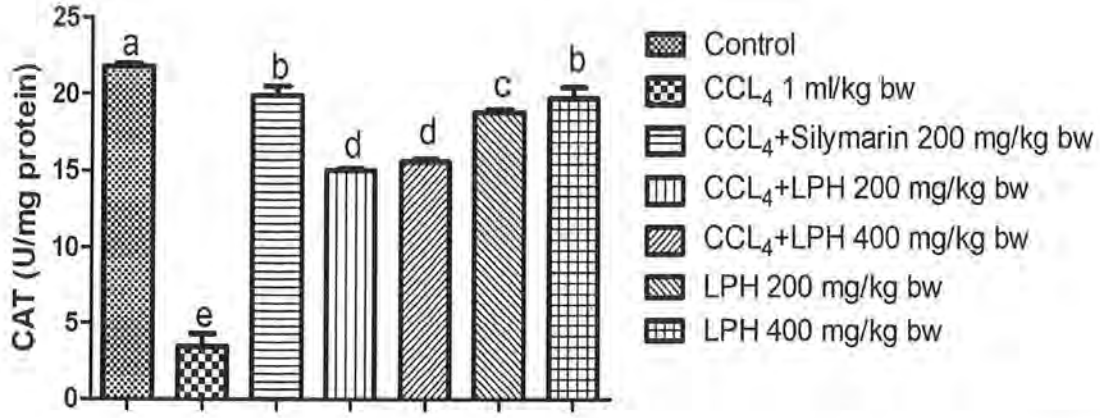

Figure 4.5 Effect of LPH on antioxidant enzymes on liver of rat; CAT (Catalase), LPH *L. pinnatifidum* hexane fraction.

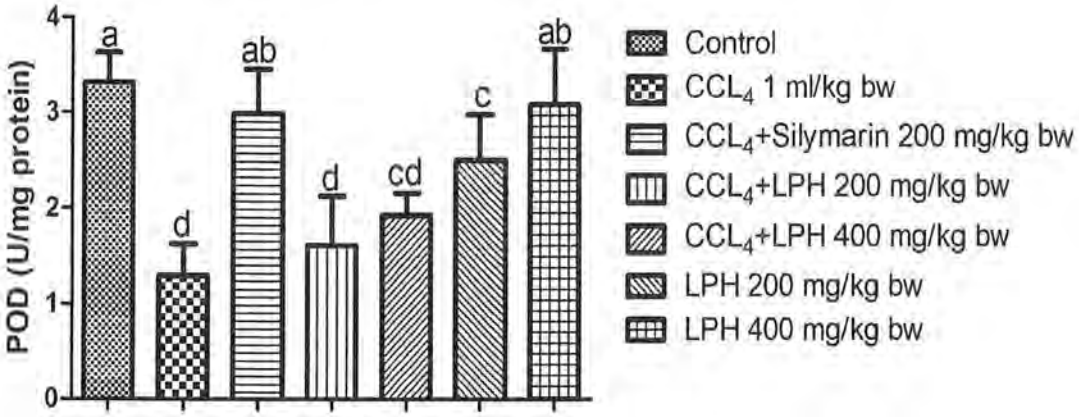

Figure 4.6 Effect of LPH on antioxidant enzymes on liver of rat; POD (Peroxidase), LPH *L. pinnatifidum* hexane fraction.

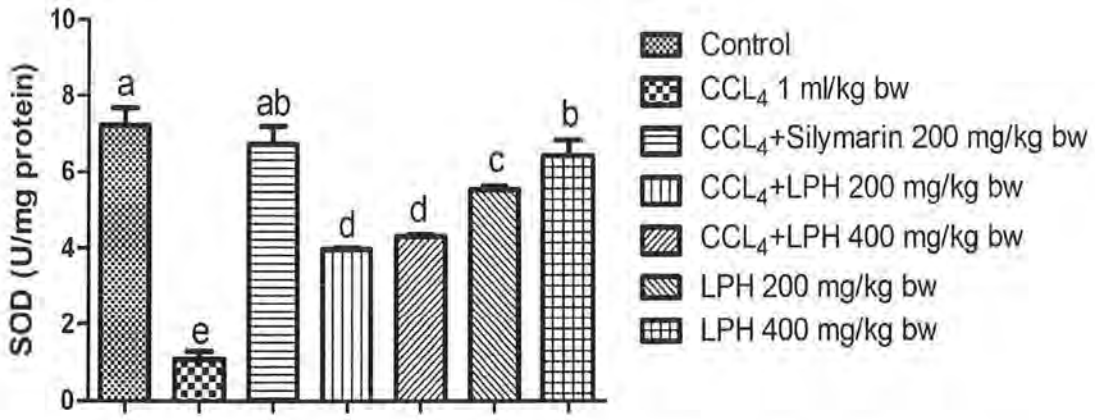

Figure 4.7 Effect of LPH on antioxidant enzymes on liver of rat; SOD (Superoxide Dismutase), LPH *L. pinnatifidum* hexane fraction.

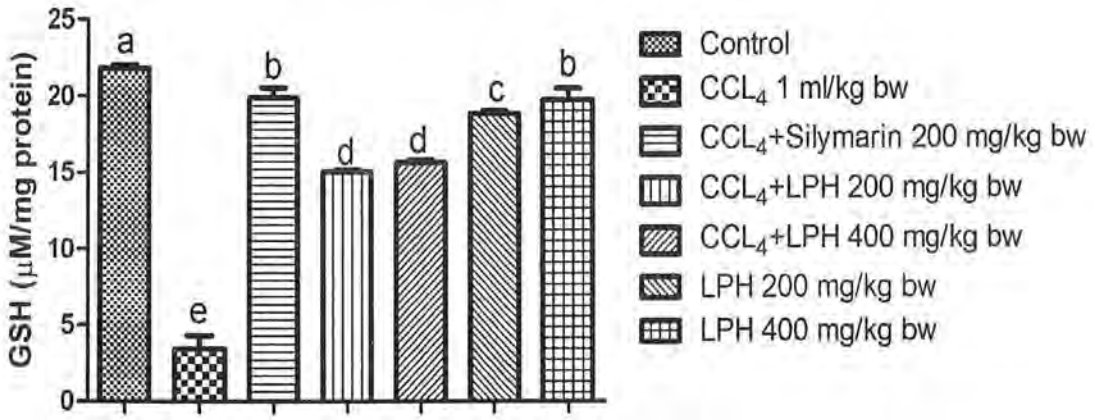

Figure 4.8 Effect of LPH on antioxidant enzymes on liver of rat; GSH (Glutathione), LPH *L. pinnatifidum* hexane fraction.

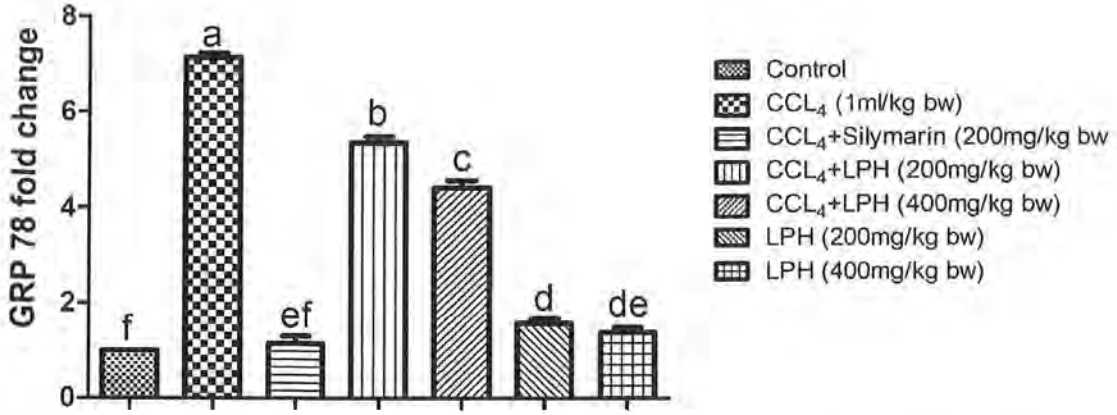

Figure 4.9 Effect of LPH on ER stress gene (GRP 78) on liver of rat; CCL_4 Carbon tetrachloride, LPH *L. pinnatifidum* hexane fraction.

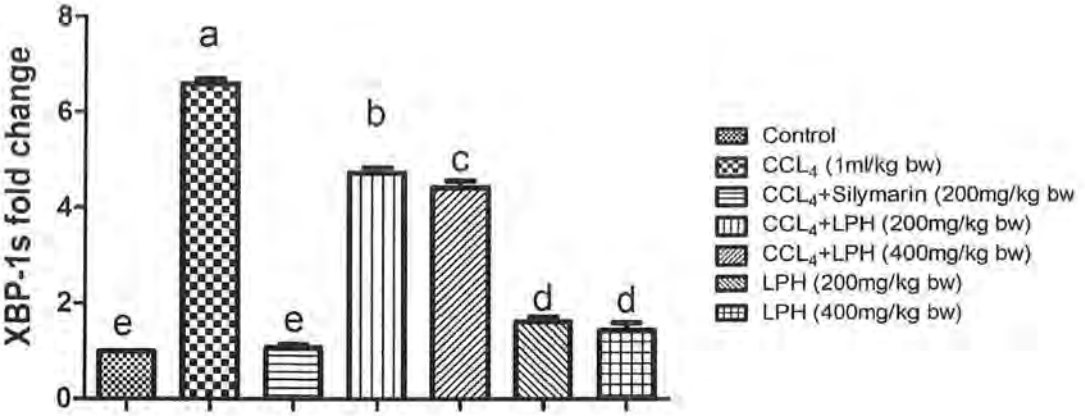

Figure 4.10 Effect of LPH on ER stress gene (XBP-1s) on liver of rat; CCL_4 Carbon tetrachloride, LPH *L. pinnatifidum* hexane fraction.

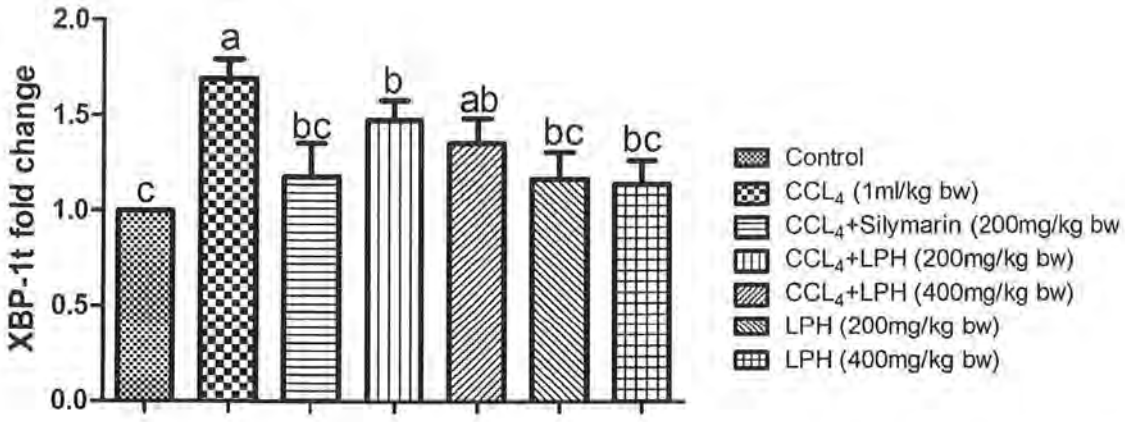

Figure 4.11 Effect of LPH on ER stress gene (XBP-1t) on liver of rat; CCL_4 Carbon tetrachloride, LPH *L. pinnatifidum* hexane fraction.

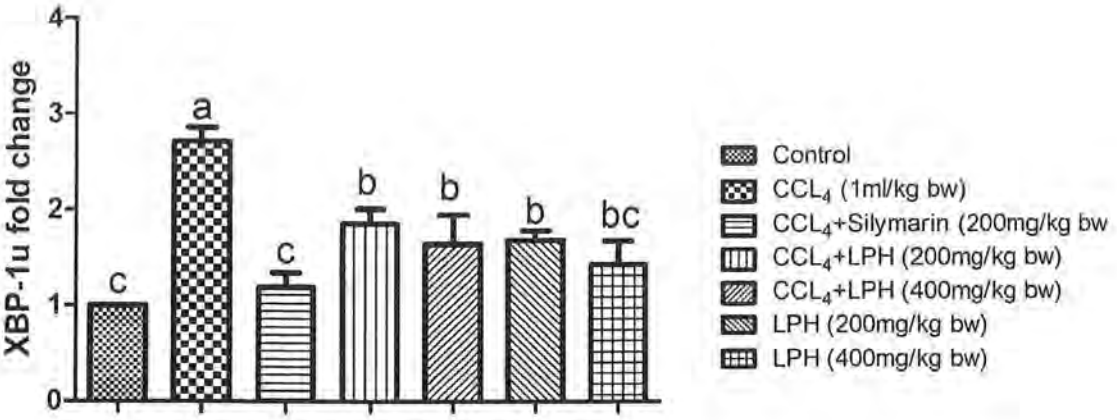

Figure 4.12 Effect of LPH on ER stress gene (XBP-1u) on liver of rat; CCL_4 Carbon tetrachloride, LPH *L. pinnatifidum* hexane fraction.

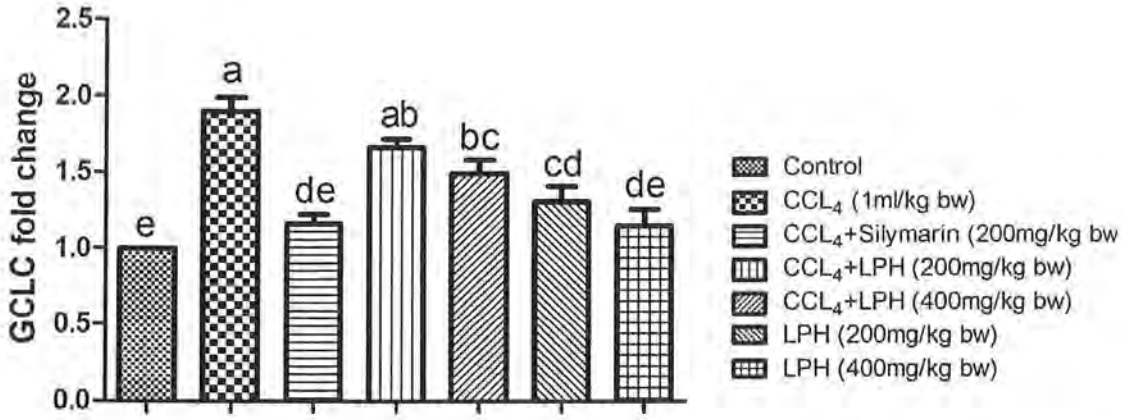

Figure 4.13 Effect of LPH on ER stress gene (GCLC) on liver of rat; CCL_4 Carbon tetrachloride, LPH *L. pinnatifidum* hexane fraction

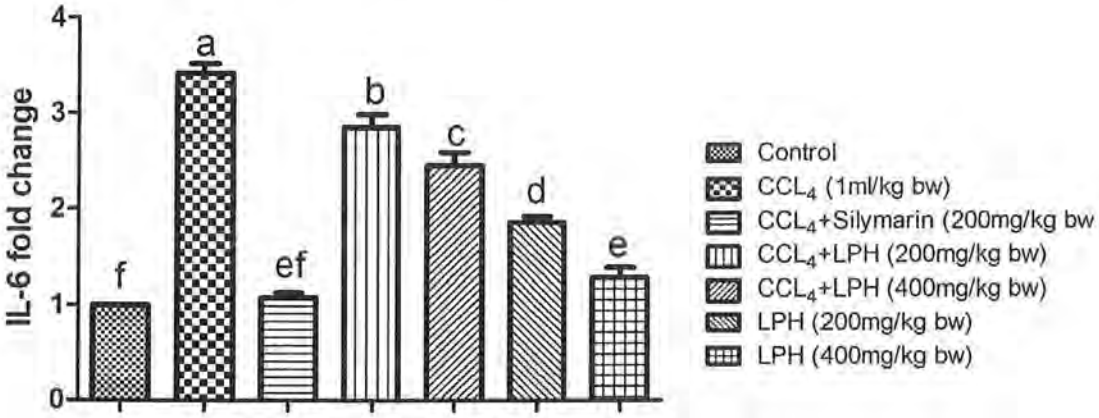

Figure 4.14 Effect of LPH on Pro-inflammatory gene (IL-6) on liver of rat; CCL_4 Carbon tetrachloride, LPH *L. pinnatifidum* hexane fraction.

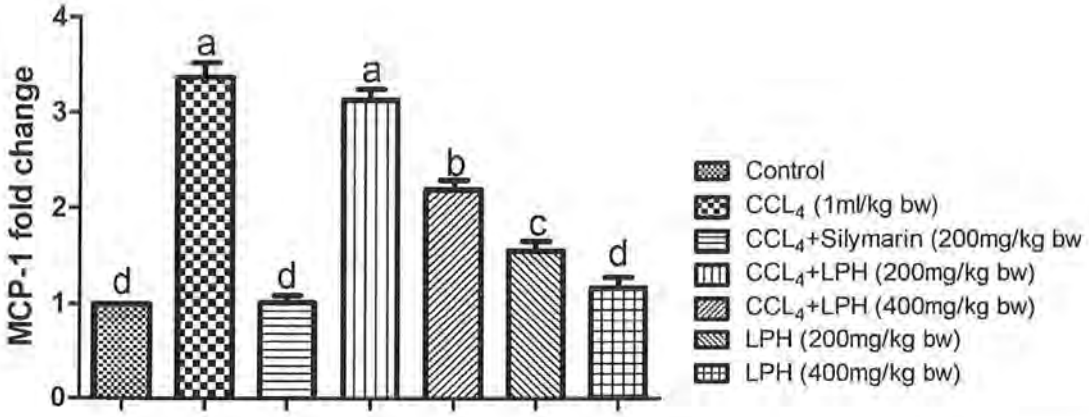

Figure 4.15 Effect of LPH on Pro-inflammatory gene (MCP-1) on liver of rat; CCL_4 Carbon tetrachloride, LPH *L. pinnatifidum* hexane fraction.

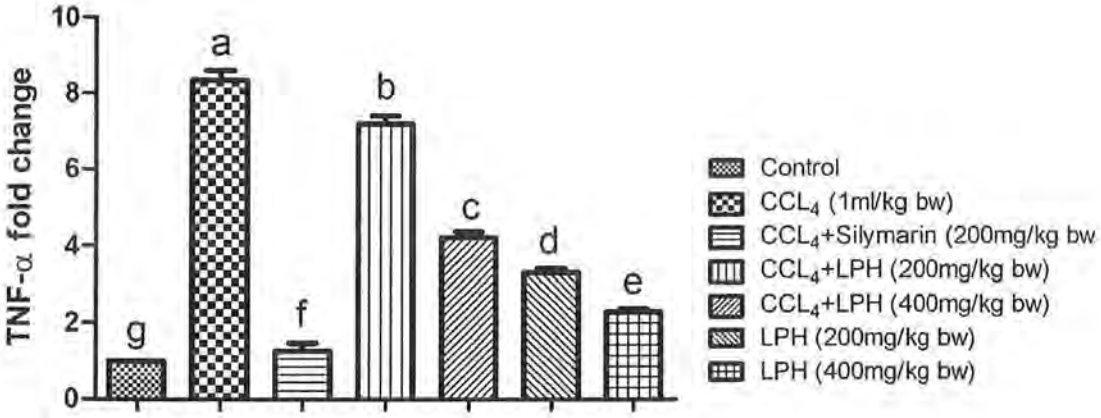

Figure 4.16 Effect of LPH on Pro-inflammatory gene (TNF-α) on liver of rat; CCL_4 Carbon tetrachloride, LPH *L. pinnatifidum* hexane fraction.

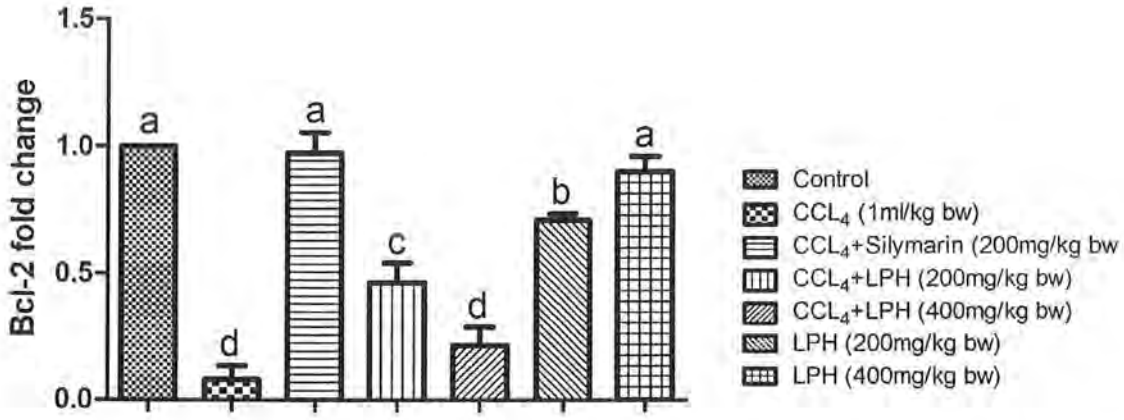

Figure 4.17 Effect of LPH on anti-apoptotic gene (Bcl-2) in liver of rat; CCL_4 Carbon tetrachloride, LPH *L. pinnatifidum* hexane fraction.

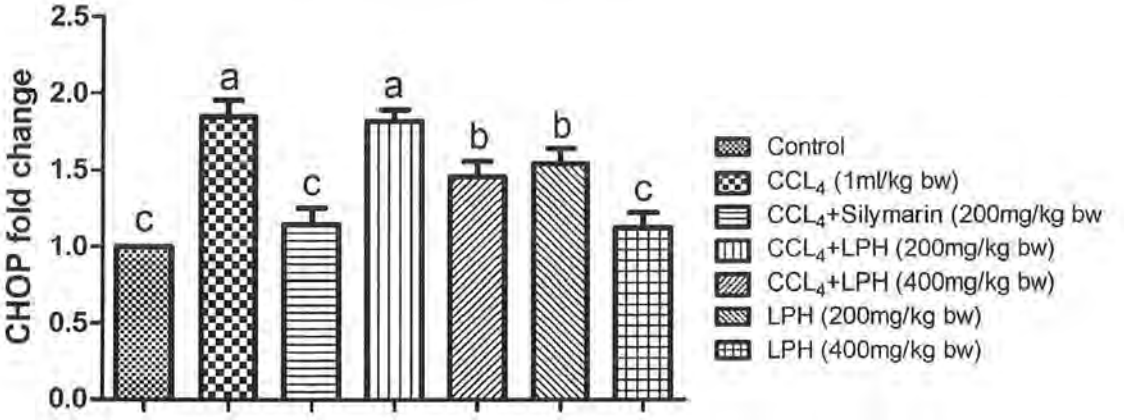

Figure 4.18 Effect of LPH on apoptotic gene (CHOP) in liver of rat; CCL_4 Carbon tetrachloride, LPH *L. pinnatifidum* hexane fraction.

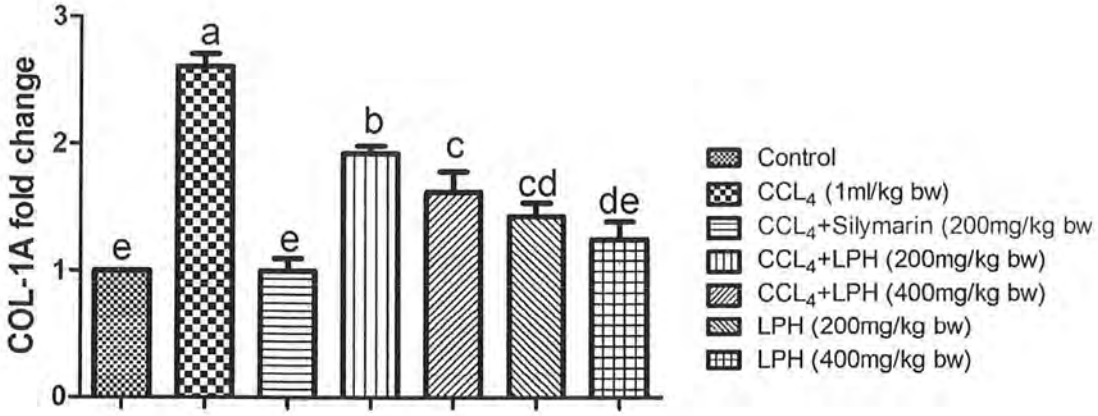

Figure 4.19 Effect of LPH on gene (COL-1A) in liver of rat; CCL_4 Carbon tetrachloride, LPH *L. pinnatifidum* hexane fraction.

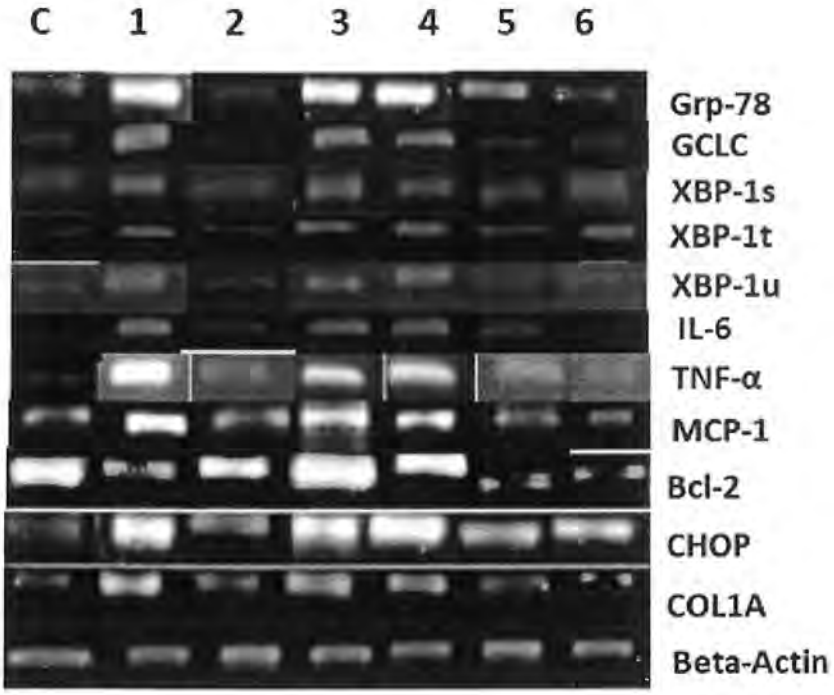

Figure 4.20 Gel electrophoresis of cDNA after RT-PCR analysis. C; Control, 1; CCL_4, 2; CCL_4+ Silymarin, 3; CCL_4+ LPH (200),4; CCL_4+ LPH (400), 5; LPH (200), 6; LPH (400)

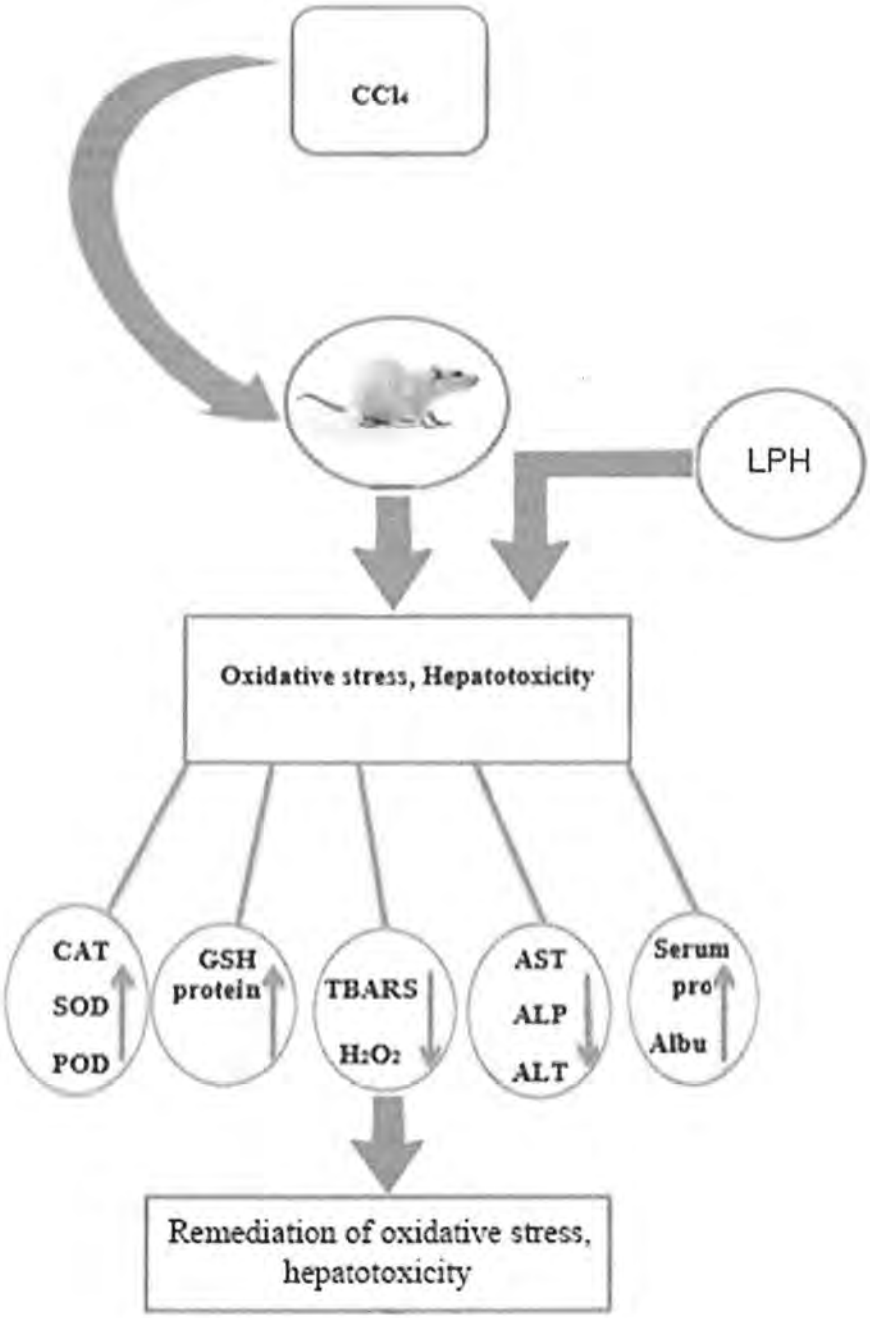

Figure 4.21: Flow diagram of remediated role of LPH against CCl_4 mediated hepatotoxicity. Pro; protein, LPH; *L. pinnatifidum* hexane fraction

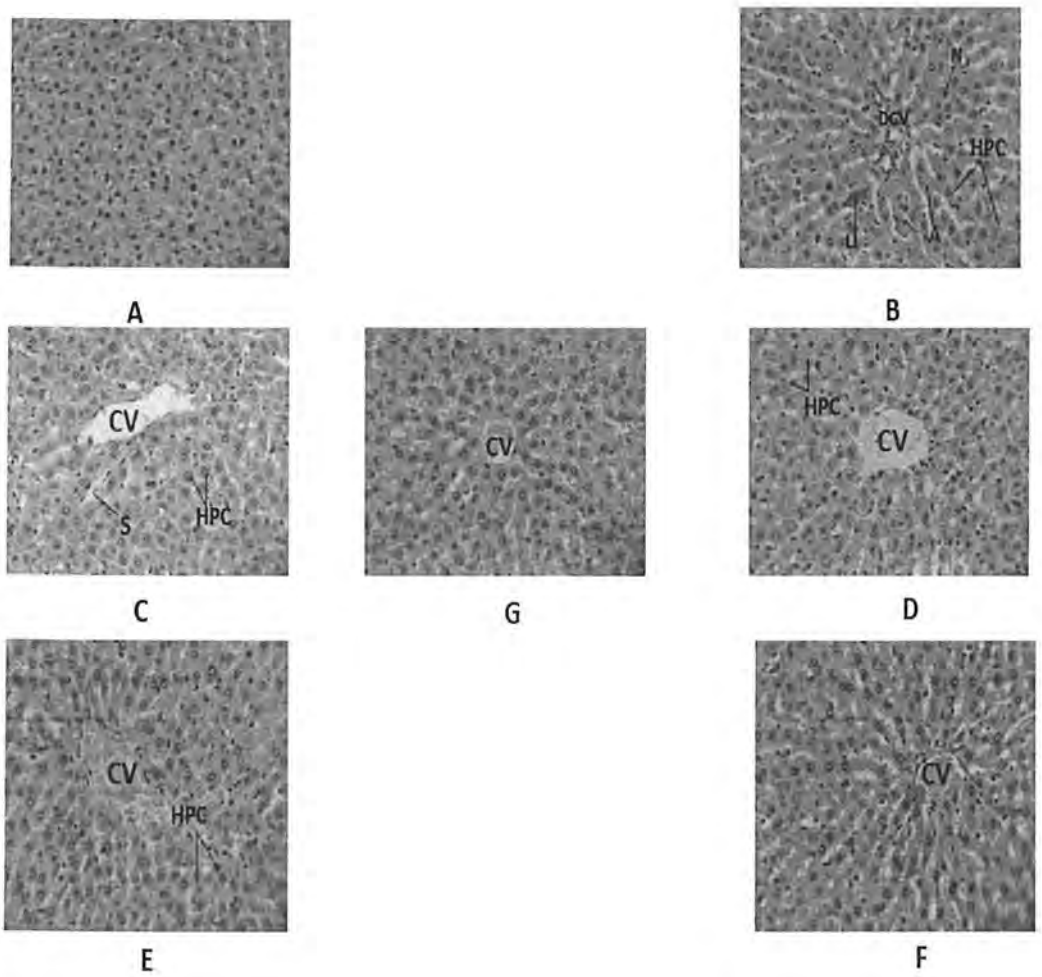

Fig. 4.22. Protective potential of LPH on liver histopathology of rats (40× magnification with hematoxylin-eosin stain). (A); Control group, (B); CCl_4 (1 ml/kg bw), (C);CCl_4 + Silymarin (200 mg/kg bw), (D); CCl_4 + LPH (200 mg/kg bw), (E); CCl_4 + LPH (400 mg/kg bw), (F); LPH (200 mg/kg bw), (G); LPH (400 mg/kg bw), CV; central vein, HPC; hepatocytes, LI; leukocyte infiltration, DCV; damaged central vein, N; necrosis, A; apoptosis of hepatocytes, LPH; *Lepidium pinnatifidum* hexane extract

DISCUSSION

From prehistoric era, human beings have knowledge about the usage of plants in treating different disorders as natural products from plants have minimum side effects (Elberry *et al.*, 2015). Several number of medicines present in today market are the formulations of obtained from medicinal plants. Current attention towards drugs and treatment from medicinal plants has made researchers curious about folk herbal medicines. In 20th century, focus has been made on the scientific estimation of folk medicines which are originated from plants for treating several ailments. Because of the effective outcomes, with probably negligible side effects in case of treatment and considerably very little prices, herbal medicines are extensively given by the physicians, even before the identification of their bio-active compounds (Levy *et al.*, 2004)

The efficacy of natural remedies for hepatic disorders has an extensive history. Although much of the recommendations on the utilization of medicinal plants for therapeutic purposes have not been done on the basis of any documented record, but some of them do have bio-active components whose antioxidant, anti-inflammatory, anti-cancerous, anti-coagulant and antiviral potential have been confirmed. Though huge amount of such medicinal plants and their use in medicinal formula have been evaluated but the results were generally disappointing. For example, the medicinal potential, in almost all of these studies, were evaluated for only small number of liver injury levels that are under clinical trials in model animals. Some causes due to which such conclusion was made are deficiency of standardized herbal medicines, inadequate randomization of placebo governed clinical trials, and scarcity of folk risk assessment of the bio-active constituents (Thyagarajan *et al.*, 2002).

Dozens of plants have been assessed for their potential to treat various number of hepatic disorders up to this point (Asadi-Samani *et al.*, 2013; McBride *et al.*, 2012). Naturally occurring bio-compounds, such as extractions of herbs, can expressively participate in restoration of the damaged liver. As reported by some authentic research on therapeutic plants, some plants such as *Silybum marianum*, *Glycyrrhiza glabra*, *Phyllanthus* species (amarus, niruri, emblica), and *Picrorhiza kurroa* have been

extensively and much of the time productively utilized for treating large number of liver diseases, employing their promising anti-oxidant potential (Tatiya *et al.*, 2012; Shukla *et al* 1991; Hu *et al.*, 2008; Mir *et al.*, 2013)

5.1. Yield of dried extracts

Plants contain several metabolic compounds that are physically and chemically active and are known as bioactive compounds. These chemical and physical activities allow biomolecules soluble in the solvents having various polarity. Solvent was extracted in downstream sequence of polarity which is hexane > chloroform > ethyl acetate > butanol. This method of separation into different fractions of a whole plant was done according to (Mir *et al.*, 2013). Aqueous fraction (LPAE) gives the highest yield (21 g), hexane (LPH, 6.2 g), chloroform (LPC, 3.5 g), butanol (LPB, 5.7 g) and ethyl acetate gives the least amount of fraction (2.35 g).

5.2. In vivo estimation of LPH

The excessive generation of free radicals including reactive oxygen species (ROS) has been associated in developing number of several acute and chronic disorders including many types of cancer, pulmonary, neurodegenerative and digestive diseases. Under normal biological conditions, the amounts of ROS are delicately controlled by antioxidants, that can be of both origins, endogenous and exogenous collectively. Deficiency in the amount of antioxidants-deficiency along with nutritive deficiencies could make persons more susceptible to oxidative damage, thus enhancing the chances of cancer development. Moreover, antioxidant protective system can be overcome in prolonged period of inflammation as in chronic obstructive pulmonary diseases, inflammatory bowel disease, and neurodegenerative disorders, cardiovascular diseases, and senescence. Specific antioxidant vitamins, for instance vitamin D, are crucial in controlling several metabolic signaling pathways leading to the appropriate performance of the organs. Supply of antioxidants in diet has been reported to regulate the deficiency of antioxidant endogenously, restoring the damage caused due to oxidative stress in several clinical trials (Liu *et al.*, 2018).).To access the protective potential of our plant, a CCl_4 exposed system is created to study about it. CCl_4 is utilized

to access the mediators causing tissue damage through the generation of free radical system. Our study has shown that treating rat models with CCl_4 for about four weeks on alternate days result in induction of inflammation and hepatic damage.

L. pinnatifidum hexane fraction is applied to access its hepatoprotective potency against hepatotoxicity generated by CCl_4 . Induction of the Silymarin is a reference drug in our experiment and the outcomes of plant fraction were somewhat similar to it.

5.3. Hepatoprotective effect of LPH

In animal models carbon tetrachloride is extensively used to produce toxicity in liver. Extensive studies have revealed that CCl_4 is a hepatotoxin which has the potential to generate oxidative stress and it is also the major cause of cellular damage by deteriorating the DNA structure, enhancing peroxidation of lipids and damaging antioxidant enzymatic system. According to a study, injecting CCl_4 intraperitoneally can cause marked increase in level of several biochemical markers such as ALP, ALT and AST. CCl_4 induction results in the damage to hepatis cells and also cause modifications in the structural characteristics cell membrane (Khan *et al.*, 2012).

Body weight is an important factor of conflicting effects of hepatotoxins and is replicated as a element that limit toxicity studies. In the current study, percent increase in weight of body of rats was reduced whereas absolute and relative liver weight was enhanced with CCl_4 administration. Our data exactly reflected the same as Lee, Shih (Lee *et al.*, 2007) who revealed that gain in absolute and relative liver weight while reduction in percent increase in body mass of rats. Although, groups treated with LPH retreat the intoxication mediated by CCl_4 and remediate the percent increase in body mass near to control group. Absolute and relative liver weight was repressed with administration of LPH to rats effected by CCL_4. These results are similar to several studies in which extracts of plant are the cause of gain in body weight and loss in liver weight in rats treated with CCL_4 (Lee *et al.*, 2015). It has been proposed that oxidative stress in liver mediated by CCl_4 results in production of toxins which helps to gain the weight of the body organ might be because of variation in metabolic processes. Moreover, therapy with plant fraction restore the liver weight close to control group by reducing the hepatotoxicity of CCl_4.

Liver has been reported as an extremely delicate gland which ceases peroxidation of lipids upon the influx of oxidative compounds. In liver CCl_4 metabolized to activate lipid peroxidation, necrotic and apoptotic cell death and deterioration of membranal structure leading to secretion of enzymatic components in blood (Huang *et al.*,2016). The first detection of hepatic damage was done by assessing ALP, ALT and AST which are generally known as crucial markers of liver and are commonly used to evaluate the plasma membrane intactness. ALP is a biomarker of endoplasmic reticulum while AST is present in mitochondrial and cytoplasmic region (Rashid and Khan, 2017). In this study, by administrating CCl_4 levels of above mentioned enzymes significantly increased which suggests that oxidative stress has caused severe harm to hepatocytes. Administration of LPH accompanied by CCl_4 has provided liver with protection against the injury produced by CCl_4, thus reducing the level of these enzymes in serum. CCl_4 also reduces the level of serum proteins which includes Globulin and Albumin, because of damaged hepatocytes membrane integrity. Control group showed the normal levels, and also the group treated with silymarin enhances the level of serum biomarkers close to the normal values. Hepatoprotective potential of plant fraction dose depends upon the concentration. High dose (400 mg/kg) has shown more effectiveness and its values are somewhat similar to the silymarin values. Our results are in accordance with one of the research done on seeds of *Phoenix dactylifera*, which also proved to have protective ability against CCl_4 induced liver damage (Abdelaziz and Ali, 2014).

Biomarkers of liver are generally present in cytoplasm of a cell and go into bloodstream when there is extensive cellular harm. Administration of Carbon tetrachloride (CCl_4) enhance the level of all the liver biomarkers enzymes in serum that is because of the injury of plasma membrane and mitochondrial membrane in liver. CCl_4 injected rats showed increased level of serum ALP, that verifies the abnormalities of liver functionality. ALP is generally involved in movement of metabolic substances across membranes inside the cell and organelles and is present in an increasing order of abundance in placental membrane, mucosal lining of ileum, kidney and liver. The increased level of ALP in serum is linked with liver cellular injury. The increased level of two other markers like AST and ALT has also been associated with liver injury. Total protein level estimation is one more criterion to detect toxic nature of substance.

Bilirubin is the final product of iron (heme) catabolic mechanism whose reaction is catalyze by an enzyme known as heme oxygenase, in the liver when worn out red blood cells are catabolized generally, due to oxidative propagation. Bilirubin estimation is considered as one of the most authentic liver function test, because it shows the capability of liver to collect bilirubin and develop it into bile. Severe nature of chronic hepatotoxicity is reflected by the high levels of total bilirubin's in serum which is due to the Increased total bilirubin's levels due to uncoupled form. As an effect of CCl_4 toxicity, the level of protein consisting of albumin and globulin diminishes in the hepatic tissue. While LPH fraction markedly results in raised serum protein levels to normal in a dose dependent manner.

Naturally our body is provided with specific antioxidant defense mechanism that includes both enzymatic antioxidants (CAT, SOD, POD) and non-enzymatic antioxidants including tocopherols, ascorbic acid, glutathione, carotenoids and many others. These antioxidants trap ROS and help in preventing damage due to oxidative stress. Catalase (CAT) has ability to neutralize hydrogen peroxide H_2O_2 via a catalytic transformation or through peroxidation metabolism. Superoxide dismutase (SOD) is a metallic enzyme in nature which catalyze the reaction of dismutation of superoxide radicals to H_2O and O_2 that have lesser toxicity. Glutathione peroxidase is an enzyme which neutralize peroxides by reducing it catalytically and thus providing protection against toxicity of ROS (El-Sayed *et al.*, 2015). Another significant antioxidant utilized in regulating redox homeostatic state of body is GSH which is one of the non-enzymatic radical scavenging enzymes. GSH is a vital enzyme for tissue susceptibility to oxidative damage as it is an ambiguous target for poisonous electrophilic substances. Decline in the levels of GSH is a strong signal of provocation of necrotic cell death in liver. decreased levels of GSH act as a vital factor in the stimulation of necrosis in liver (Abdel-Moneim *et al.*, 2015). All the above mentioned antioxidant enzymes and GSH level in cells were reduced markedly in CCL_4 treated group in our experiment, which have caused cellular injury in liver cells through deposition of ROS. Administration of LPH along with CCl_4 has significantly restored antioxidant enzyme values near to the control ones . The elevation of GSH level could be because of GSH revival or de novo production. It also confirms the statistic that bioactive components present in LPH are

efficient in increasing the levels of antioxidants as well as proved to be effective in combating the infectious diseases. Our findings are in accordance to (El-Sayed *et al.*, 2015) who also testified that the reduction in the levels of antioxidants enzymes in pathological state and recovery towards control after treating the animal model with plant extract.

The job of nitric oxide (NO) in oxidative stress is very vital as the increased levels of NO have been affiliated with oxidative injuries through generating lipid peroxides and nitrite free radicals which causes the induction of inflammation. It is generated by endothelial cells, kuppfer cells and hepatocytes as a response to tissue damage and inflammation caused by hepatotoxin like CCl_4 (Al-Olayan et *al.*, 2014). In this present study nitrite levels of liver were increased in rats receiving the treatment of CCl_4. This elevation was significantly reduced when LPH was administered which suggest the anti-inflammatory aptitude of the plant extract. Liver injury in the case of rats with CCl_4 treatment can also be detected by the increased levels of TBARS which are outputs of lipid peroxidation taking place in cell membranes. Chloride (Cl^-) ion derivatives generated in CCl_4 metabolism lead to damages to cell membranes. These ions binds with poly unsaturated fatty acids (PUFA) found in the membranes, results in peroxidation of them and generate extremely reactive carbonyls i.e. TBARS that are one of the major cause of hepatic injuries (Gite *et al.*, 2014). Our study reported elevated levels of TBARS, H_2O_2, nitrite and reduced levels of tissue proteins in liver in CCl_4 treated animal group. Administration of LPH along with CCl_4 treatment animal group showed decrease in their levels and elevated tissue proteins. Moreover, group treated with LPH returned the levels of these substances near to that of control ones. The reduced levels of TBARS might be due to increased level of antioxidant enzymes that lead to detoxification of these injurious constituents. These findings are in coherence with the studies directed by Suzek, Celik (Suzek *et al.*, 2016).

Aberrations in ER function has highly affected the release of apolipoproteins. Earlier studies have testified the significance of proteins for the persistence of hepatic cells against CCl_4 related oxidative stress (Oda et al., 2003). Hepatocytes abundantly contain both rough and smooth endoplasmic reticulum because they have to perform large number of metabolic activities such as biosynthesis of cholesterol , production and

secretion of certain plasma proteins, lipoproteins most prominently very low density lipoproteins (VLDL) and hepatotoxic metabolic reactions (Malhi and Kaufman, 2011). When the cell is exposed to xenobiotics like CCl_4, which leads to the production of free radicals (ROS) that upset synthesis and folding of proteins by ER. During the general procedure of protein production, the proper folding of freshly produced proteins and breakdown of unfolded proteins is controlled by molecular chaperones in the ER. Moreover, a defensive response activated by cells i.e. unfolded protein response (UPR) is activated by the deposition of misfolded or mal-folded proteins. This response causes reduction in the levels of irregular proteins via induction of several signaling pathways which cease translation of proteins and increase the generation of chaperons. Under the physiological states where the accumulation of unfolded proteins takeovers the above mentioned modifications, ER stress is resulting in the initiation of production of caspases and ultimately apoptotic cell death occur (Rutkowski *et al.*, 2008) GRP78 also called as binding immunoglobulin protein (Bip) is the key controller of ER stress response because it reacts and deactivate stress indicators present in the lining of ER. The present study revealed that intoxicated rats with CCl_4 showed marked increase in the expression of GRP78 to some folds. Treatment with *L. pinnatifidum* hexane fraction significantly decrease its expression on receiving low and high LPH dose revealed more prominant results by altering its expression close to that of control ones. This result is exactly the same as that of the findings of (Lee *et al.*, 2014)who showed that in hepatocytes,CCl_4 results in the swelling of rough endoplasmic reticulum, thus employing ER stress that causes elevation in the expression of GRP78 and XBP-1. GRP78 also binds with a transmembrane protein IRE-1α (inositol requiring enzyme 1α) which get activated by phosphorylating automatically and results to form spliced variants of an intron from XBP-1 and thus generating XBP-1 transcription factor. That XBP-1 s transports to nucleus where it reacts with various co-mediators to regulate the expression of some genes governed in ER-associated degradation (ERAD) (Malhi and Kaufman, 2011). One of the crucial role of ERAD pathway is to degrade all the unfolded or misfolded proteins present in the ER. As a result of ER stress induced by CCl_4, the expression of XBP-1 s was elevated many folds with a decreased expression obtained in the rat groups getting treatment along with CCl_4 and plant extract in a dose dependent manner. Also, this expression was generally controlled in animals getting the plant extract alone which indicates that the

polyphenols present in the plant fraction could be the reason of normalizing gene expression and ordinance of ER homeostatic mechanism. Another significant consequence was that expression of GCLC which is a one of the master regulator of GSH is elevated in animals which received treatment of LPH delivering the proof that LPH provides protection against CCl_4 induced oxidative damage by increasing the expression levels of antioxidants.

One of the most significant transmembrane protein of UPR signaling pathway is PKR like ER protein kinase (PERK) which perform vital role in regulating ER stress by reducing the chunk of protein level and cell cycle arrest. This kind of PERK associated translational halt is essential for the provocation of nuclear factor-κB (NF-κB), c-jun N terminal kinase (JNK), IL-6 and TNF-α. As a result of ER stress inducted by prolonged exposure to CCl_4, deposition of unfolded abnormal proteins intracellularly, takes place which could be accountable for various disorders linked with cellular damage and initiation of inflammatory response (Rutkowski *et al.*, 2008). Pro-inflammatory mediators i.e. cytokines including TNF-α, IL-6 and chemokine MCP-1 are the important regulators in causing hepatic inflammation as MCP-1 is the cause of stimulation of more blood cells to be moved to the site of liver damage. Our case reported relatively elevated expression of TNF-α, IL-6 and MCP-1 in rats receiving the treatment of CCl_4 delivering a hint of inflammation as they have been revealed in liver fibrosis earlier similar studies (Huang *et al.*, 2016; Zheng *et al.*, 2008; Alam *et al.*, 2018) . Nevertheless, expression of these inflammatory genes was regularized by LPH in a dose dependent manner. While the original anti-inflammatory procedure of LPH is not evidently identified however it could be presumed that polyphenols and other bioactive components of LPH are accountable for inhibiting TNF-α and IL-6 (Al-Rasheed *et al.*, 2015). Our data is similar to some other findings where polyphenolic compounds from medicinal plants revealed promising anti-inflammatory potential (Khalifa *et al.*, 2018).

Induction of ER-stress for longer period of time causes CHOP (CCAAT-enhancer-binding protein homologous protein) to express through major three UPR pathways (IRE1α, PERK and ATF6) (Tabas and Ron 2011; Ron and Walter 2007) and then CHOP activation causes induction of expression of certain pro-apoptotic genes like

Bim and Ero1l (Puthalakath et al. 2007; Li et al. 2009), and also causes repression of antiapoptotic genes i.e. Bcl-2 (McCullough et al. 2001). Our study revealed that in the case of administration of CCL_4,up-regulation of CHOP and repression of Bcl-2 genes takes place which symbolizes apoptotic cell death. However, their behavior is normalized to that of control on the administration of LPH in a dose dependent manner. These results are in accordance with the earlier studies (Sekine *et al.*, 2011; Lu *et al.*, 2012).

There are several types of collagen synthesized in animals and consists of subunits which have been identified so far COLlA(1) and COL2A(I) subunits that are needed for proper fibrous conformation of type I collagen abundantly found in tissues such as liver (Vuorio and De Crombruqqhe, 1990) ain diseased states such as several stimuli i.e. high intake of alcohol and infectious agents, the elevated level of collagen synthesis taes place in liver and might results in liver fibrosis and cirrhosis (Bissell *et al.*, 1990). Administration of CCL_4 causes COLlA level to increase many folds in hepatocytes (Itoh *et al.*, 1992). Administration of LPH along with CCl_4 treated animal group showed reduced in the expression level of gene. Moreover, group treated with LPH restored its expression level near to that of control group.

The histopathology of liver provides a straight criterion of assessing CCl_4 induced hepatocellular injuries as well as attributing our extract as efficiently defensive in detoxification of lethal effects due to hepatotoxicity of CCl_4. Histological investigation of rats treated with CCl_4 displayed extreme notch of disruptions in hepatic cells which includes cellular hypertrophy, intrusion of inflammatory cells, intrusion of central lobule and sinusoidal obstruction. Administration of CCL_4 along with LPH low and high dose corrected these losses. Treatment with LPH low and high dose alone have not shown any morphological changes in liver anatomy . Similar results have been revealed by Chen, Zhan (Chen *et al.*,2017) who showed that the protective aptitude of *Schisandra lignan* extract against CCl_4 endured hepatic damage. The protective potency of LPH could be accredited because of the presence of polyphenols and other bioactive constituents which consist of potential antioxidant potency thus preventing tissue deterioration by preventing peroxidation of lipids in liver.

5.4. Conclusion

On the basis of polarity of fractions of *Lepidium pinnatifidum* different biochemical activities are performed which shows the medicinal potency of plant in certain fields

- Methanolic extract and its resultant fractions were detected to be present in various amount, which reveals the striking characteristics of phytochemicals, their utilization and allocation.
- Phytochemical analysis of several fractions of *Lepidium pinnatifidum* showed the occurrence of bioactive constituents such as flavonoids, alkaloids, cumarins, saponins, tannins and terpenoids that was a validation of pharmacological aptitude of *Lepidium pinnatifidum* .
- Ethyl acetate fraction displayed highest level of phenolic and flavonoid constituents.
- *In vivo* assays revealed that CCl_4 deteriorate crucial components present inside the cell like DNA, lipids and proteins, along with this, it mediate intoxication in several organs such as liver, heart and brain etc. Plant dose has restored the percent increase in body weight as well as relative liver weight near to control. High dose of *Lepidium pinnatifidum* restored the toxicity of liver mediated by CCl_4 and repressed the ALP, ALT, AST, GSH and serum protein level and regularize the antioxidant enzymes, usual TBARS and protein level, normal and consistent microanatomical outline of liver tissue.
- *Lepidium pinnatifidum* also revealed protective effect by restoring the mRNA expression levels of ER stress markers, pro-inflammatory mediators and apoptotic genes against the intoxicated effects of CCl_4.

5.5. Future Perspective

Current study revealed the hepatoprotective effects of *Lepidium pinnatifidum*, even then further effective research and experimentation is required in identification and extraction of certain biochemical constituents which could have pharmacological ability. Moreover, advanced research techniques and tools are needed in order to characterize and develop new herbal drugs.

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