HEPATOPROTECTIVE EFFECTS OF MEDICINAL PLANTS EXTRACTS ON CARBON TETRACHLORIDE (CCl_4) INDUCED LIVER DAMAGE IN MICE

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By

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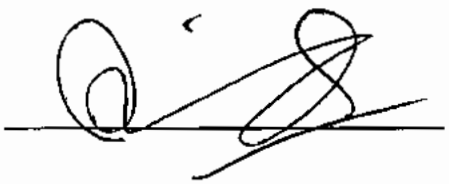

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Submitted to International Islamic University, Islamabad, Pakistan in partial fulfillment of the requirements of the degree of Doctor of Philosophy (Biotechnology).

In the name of Allah,

Most gracious, most merciful.

All praise be to Allah

Lord of all the worlds,

Most of beneficient, ever merciful,

Lord of the day of judgements

You alone we worship, and to you

Alone we turn for help.

Guide us (O Allah) to the path that is straight,

The path of those you have blessed,

Not of those who have earned your anger,

Nor those who have gone astray.

Al Quran: The opening Chapter

DEDICATION

This work is dedicated

to

The loving memory of my late parents

who

Cherished this dream for me.

Also

to

my

Wife

Daughter

Sons

Brothers

Sisters and

my Teachers

DECLARATION

It is certified that work done on this Ph. D Biotechnology research thesis is purely conducted by me. I carried out the experimental work described in this thesis in Biotechnology Lab, Department of Biological Sciences, International Islamic University Islamabad (IIUI) Pakistan, Animals House Lab, National Institute of Health (NIH) Islamabad and the School of Pharmacy and Pharmaceutical sciences, University of Dublin, Ireland. All the material is prepared by myself and has not been copied from anywhere; however, some test and figures have been used which are properly referenced. The conclusions are my own research after numerous discussions with my supervisor. All the assistance and help received during the course of research have been duly acknowledged.

Date _____

Muhammad Zakriyya Khan

I certify that the above statement is correct.

Dr. Muhammad Arshad Malik

Supervisor

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ABBREVIATIONS

Abbreviation	FULL FORM
VCME	*Violacanescens* methanolic extract of flowers
DRME	*Dryopteris ramosa* methanolic extract of leaves
GWME	*Geranium wallichianum* methanolic extract of rhizome/roots
EPME	*Eleagnus parvifolia* methanolic extract of fruits
RBME	*Rosa brunonni* methanolic extract of flowers
AAME	*Acconogonon alpinium* methanolic extract of roots
ODME	*Oxyria digyna* methanolic extract of shoots
FNME	*Fragaria nubicola* methanolic extract of whole plant
TSME	*Thymus serphyllum* methanolic extract of shoots
PMME	*Primula macrophylla* methanolic extract whole plant
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
ALP	Alkaline phosphatase
TB	Total bilirubin
DPPH	2,2-Diphenyl-1-Picrylhydrazyl
GSH	Glutathione
TAA	Thioacetamide
NASH	Non-alcoholic Steatohepatitis

CLD	Chronic liver diseases
NAFLD	Nonalcoholic fatty liver diseases
ROS	Reactive oxygen species
HSH-Px	Glutathione peroxidase
SOD	Superoxide dismutase
RNS	Reactive nitrogen species
WHO	World Health Organization
AJ&K	Azad Jammu and Kashmir
KPK	Khyber Pakhtunkhwa
CCl_4	Carbon Tetrachloride
NKC	Natural killer cells
TNF	Tumor necrosis factors
g	Gram
mg/kg	milligram per kilogram
mg	Milligram
µg/mL	Microgram per milliliter
ng	nano gram
µg	micro gram
µg/mg QE	microgram per milligram quercetin equivalent
HAV	Hepatitis A Virus

HBV	Hepatitis B Virus
HCV	Hepatitis C Virus
CAM	Complementary and Alternative Medicines
TP	Total proteins
RP-HPLC	Reversed-Phase High Performance Liquid Chromatography
SGPT	Serum glutamate pyruvate transaminase
SGOT	Serum glutamate oxaloacetic transaminase
HE	Hematoxylin & Eosin
PMNH	Pakistan Museum of Natural History
ME	Methanolic extract
NIH	National Institute of Health
HPLC	High Performance Liquid Chromatography
OECD	The Organization for Economic Co-operation and Development
SD	Standard drug
IBBC	Institutional Bioethics and Biosafety Committee
AA	*Acconogonon alpinum*
EP	*Eleagnus parvifolia*
GW	*Geranium wallichianum*
HF	n-Hexane fraction
CF	Chloroform fraction

EF	Ethyl acetate fraction
BF	n-Butanol fraction
AF	Aqueous fraction
IC_{50}	Half maximal inhibitory concentration
"Ac"	Absorbance of negative control
"As"	Asorbance of test sample
µg/mg GAE	Microgram Per-Milligram Gallic Acid Equivalent
AA	Ascorbic acid
QE	Quercetin equivalent
TPC	Total phenolic content
TFC	Total flavonoid content
ANOVA	Aanalysis of Variance
LD_{50}	Lethal dose
mg/dL	milligram per deciliter
%	Percent
<	Less than
>	Greater than
°C	Degree centigrade
PCM	Paracetamol
CV	Central vein

SS	Sinusoidal spaces
H	Hepatocytes
K	Kupffer cells
F	Focal Necrosis
FD	Fatty degeneration
PV	Portal Vein
GWHF	*Geranium wallichianum* n-hexane fraction
GWCF	*Geranium wallichianum* chloroform fraction
GWEF	*Geranium wallichianum* ethyl acetate fraction
GWBF	*Geranium wallichianum* n-butanol fraction
GWAF	*Geranium wallichianum* aqueous fraction
EPHF	*Eleagnus parvifolia* n-hexane fraction
EPCF	*Eleagnus parvifolia* chloroform fraction
EPEF	*Eleagnus parvifolia* ethyl acetate fraction
EPBF	*Eleagnus parvifolia* n-butanol fraction
EPAF	*Eleagnus parvifolia* aqueous fraction
AAHF	*Acconogonon alpinum* n-hexane fraction
AACF	*Acconogonon alpinum* chloroform fraction
AAEF	*Acconogonon alpinum* ethyl acetate fraction
AABF	*Acconogonon alpinum* n-butanol fraction

AAAF	*Acconogonon alpinum* aqueous fraction
LCMS	Liquid Chromatography Mass Spectrometry
QTOF	Quadrupole-Time of Flight
LOQ	Limit of quantification
µL	Microliter
mg/mL	milligram per mililiter
EC_{50}	Half maximal effective concentration
LOD	Limit of detection
S/N	Signal-to-noise
MS/MS	Tandem Mass Spectrometery
m/z	Mass-to-charge ratio

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Muhammad Zakryya Khan

ABSTRACT

Plants have medicinal importance for pharmaceutics that is why they are known as treasure house of medicines all around the world. *Viola canescens*Wall, *Dryopteris ramosa* C. Hope, *Geranium wallichianum* D. Don ex sweet, *Eleagnus parvifolia* Wall ex. Royle, *Rosa brunonii* Lindl, *Aconogonon alpinum* All Shur, *Oxyria digyna* (L) Hill, *Fragaria nubicola* Hook F, *Thymus serphyllum* Ronnigerand *Primula macrophylla* D. Don are importantly considered traditionally used folk medicines and generally used in the Poonch Division of Azad Jammu and Kashmir, Pakistan, treating different diseases. These plants have not been assessed scientifically for their hepatoprotective effects. In this study, the selected medicinal plants were screened to evaluate theirsafety and hepatoprotective activity in the mice. Daily administration of crude extract (2000 mg/kg) of these plants in respective groups (10 groups) of mice for14 days did not show any behavior change in the mice showing that LD_{50} (in mice) of each selected plant was beyond the 2000 mg/kg. To assess the hepatoprotective effect of crude methanolic extract of selected plants, animals were divided in 23 groups (n= 6). Group one (G-I) was normal control while G-II to G-XXIII were injected with 25% CCl_4 (1mL/kg) intraperitoneally with 2 days interval for 13 days and then treatment of G-III to G-XXIII with the orally administered Silymarin (100 mg/kg) and low and high dose (200 mg/kg/d and 400 mg/kg/d) of methanolic extracts of *V. canescens* flowers (VCME), *D. ramosa* leaves (DRME), *G. wallichianum* rhizome/roots (GWME), *E. parvifolia* fruits (EPME), *R. brunonni* flowers (RBME), *A. alpinium* roots (AAME), *O. digyna* shoots(ODME), *F. nubicola* whole plant (FNME), *T. serphyllum* shoots (TSME) and *P. macrophylla* whole plant (PMME) respectively. There was significant ($P < 0.01$) decrease in the liver biochemical parameters (ALT, AST, ALP & TB) with low dose extract i.e. 200 mg/kg and this decrease was further improved with the high dose extract (400 mg/kg) in dose dependent manner of each selected plant extract and hepatoprotective effect was further confirmed by histopatholical analysis of mice livers. The most active hepatoprotective plants extract i.e.*G. wallichianum* (A), *E. parvifolia* (B) and *A. alpinum* (C) were subjected to bio-guided treatments. Five partitioned fractions of each plant (*n*-hexane, chloroform, ethyl acetate, n-butanol and an aqueous fraction) were tested for hepatoprotective effects

against CCl_4 induced toxicity in mice in 33 groups (n= 6) and DPPH antioxidant activity. The liver marker enzymes were reduced significantly ($P < 0.01$) with the treatment of different fractions of *G. walichianum*, *E. porvofolia*, and *A. alpinum*. Comparison of effect of fractionsshowed that *G. wallichianum* ethyl acetate fraction (GWEF), *G. wallichianum* butanol fraction (GWBF) and *G. wallichianum* aqueous fraction (GWAF) showed highest protective effects in dose dependent manner. Similarly, *E. parvifolia* ethyl acetate fraction (EPEF) and E. *parvifolia* aqueous fraction (EPAF) are most active for hepatoprotective effect. Comparison of effect of *A. alpinum* partitionedfractionsshowed that *A. alpinum* ethyl acetate fraction(AAEF), *A. alpinum* n-butanol fraction (AABF) and *A. alpinum* aqueous fraction (AAAF) are most active for hepatoprotective effect in the dose dependent manner against CCl_4 induced toxicity in mice as compared to toxicant control group. Results of antioxidant activity for DPPH assay showed that in case of *G. wallichianum*; there was highest antioxidant activity revealed by GWAF followed by GWEF and GWBF (IC_{50} values 29.92 and 38.06 and 40.26 µg/mL respectively). Similarly, *E. parvifolia* revealed the highest activity of EPAF following by EPEF (IC_{50} values 32.07 µg/mL and 38.99 µg/mL) and *A. alpinum* of AAAF followed by AAEF and AABF (IC_{50} 25.51 µg/mL and 30.20 µg/mL and 42.19 respectively) as compared to standard ascorbic acid (IC_{50} value 31.90 µg/mL). Hepatoprotective and antioxidant activity is attributed to 14 phenolic and flavonoids (gallic acid, chlorogenic acid, caffeic acid, ferulic acid, epicatechin, hyperoside, rutin, apigenin-7- o-glucoside, luteolin, naringenin, fisetin, benzotriol, chrysin and apigenin) in *G.wallichianum*,*E. parvifolia* (except chlorogenic acid, rutin and fisetin) and *AAlpinum* identified by HPLC and seven (7) of them (gallic acid, caffeic acid, chlorogenic acid, epicatechin, hyproside, ruitn and apigenin) further confirmed by LCMS in addition to gallic acid dimer, quercitrin, kaempferol, protocatechuic acid, vanillic acid (in *A. alpinum*), syringic acid, quinic acid (in *G. wallichianum*), catechin and quercetin (in *E. parvifolia* except kaempferol, syringic acid and vanillic acid) phytoconstituents identified by LCMS. Over all our studied plants showed significant hepatoprotective activity in liver damaged mice.

INTRODUCTION

1. INTRODUCTION

1.1. Liver Role and Its Complications

Liver in our body is an important organ comprising four lobes of unequal size (Cortan *et al.*, 2005). Liver plays an important role for normal maintenance of metabolic activities and regulation of physiological process in our body (Sjogren *et al.*, 2010). The most important functions of liver include synthesis of blood clotting factors, elimination of toxic substances from body, detoxification of blood, regulation and synthesis of essential hormones (Heidelbaugh and Brudery, 2006). In general, all biochemical pathways from growth to fight are involved in the liver (Vankat *et al.*, 2015). Liver is most exposed organ to xenobiotics and it plays major role for enzymatic conversion of toxic metabolites such as lipophilic pharmacologically active molecules, into water soluble metabolites that are excreted through urine (Mroueh *et al.*, 2004; Saoudi and Feki, 2012; Kumar *et al.*, 2013).

Because of strategic location and multiple functions, many diseases also affect the liver. Liver inflammation is caused by different agents. Hepatotoxins; such as xenobiotics, drugs overdose, alcoholism and industrial chemicals damage the liver (Atiq *et al.*, 2004; Kashawa *et al.*, 2011). High concentration of chemicals and drugs reach to liver where they accelerate its damages. There are about 900 drugs, which are thought to cause liver damages that is why, those drugs have been withdrawn from the markets. Some therapeutic medicines when taken overdose and organic solvents, cause injuries to the body organs including liver (Kashawa *et al.*, 2011). Drugs induced toxicity is variable which ranges from asymptomatic increase in liver enzymes to hepatic malfunction. Continuous exposure to toxins of environment and abuse drugs habits such as overdose of paracetamol (acetaminophen), causes liver damage. Metabolism of acetaminophen causes oxidative stress and reduction in glutathione (GSH), which is strong antioxidant in the body (Russmann *et al.*, 2009). Alcoholism leads to hepatitis, cirrhosis and alcoholic liver diseases (Vankat *et al.*, 2015). Both acute and chronic hepatitis (liver inlamation), cirrhosis, hepatosis (non-inflammatory

diseases) and jundice are important classified liver disorders (Alshawsh*et al.*, 2011; Kumar *et al.*, 2013). Numbers of other liver complications comprise; liver cancer, liver failure, bacterial peritonitis, hepatic encephalopathy, hemotochromatosis, ascites and hepatitis C (Yash, 2000).

In developed countries, principal causative factor of liver disorder is over consumption of alcohol and viral-induced chronic liver diseases while environmental toxins, laboratory chemicals i.e. carbon tetrachloride (CCl_4), parasitic infectious diseases, hepatitis B virus, hepatitis C virus, chemothraputic drugs, thioacetamide (TAA) and excess use of antibiotics cause liver disorders in developing countries frequently (Schuppan and Afdha., 2008; Rehm, 2013). In patients who have diabetes mellitus, obesity and metabolic syndrome are observed to suffering from fatty livers in developed and developing countries. Due to lack of physical activities and overeating habits, nonalcoholic steatohepatitis (NASH) has become worldwide problem (Okazaki *et al.*, 2014). Chronic liver diseases (CLD) which are global health problem are neglected and in next decade, the NASH will increase certainly (Marcellin *et al.*, 2018). In Western and developing countries, liver cirrhosis and liver injury caused by drugs, induction is counted as ninth leading cause of death (Saleem *et al.*, 2014). Liver cancer associated with cirrhosis is about ninety percent to reported (Okazaki *et al.*, 2001). Alcoholic cirrhosis in patients has serious complications and mortality in Asian pacific region (Yang, 2016). In Asia the nonalcoholic fatty liver diseases (NAFLD) prevalence is 40% in some countries and risk factors are similar to Western countries. There is also evidence of interaction of NAFLD, Hepatitis B and hepatitis C virus in Asian countries (Seto and Yuen, 2017). In Pakistan, 20% of patients that use medicines to treat tuberculosis (T.B) develop liver injury due to side effects of medicines (Sana *et al.*, 2010).

In addition, reactive oxygen species (ROS) such as superoxide anion, hydroxyl radicals and hydrogen peroxide (H_2O_2) that are unstable compounds having unpaired electrons in their outer most shell, also play their vital role to cause liver damage. These free radicals are produced due to anaerobic respiration or from exogenous sources. Reaction of biological molecules i.e. proteins, lipids and deoxyribonucleic acids (DNA) with free radicals cause oxidants and antioxidants imbalance (Agbor and Nagogang, 2005). Antioxidant system

reduces correlation between reactive oxygen species (ROS) and degenerative diseases such as arthritis, cirrhosis, cancer, Alzheimer and aging. Reactive oxygen species are regularly scavenged by antioxidant system. An imbalance between ROS and antioxidant protective mechanism in the body during metabolism leads to principal cause of liver disorders (Morenoa and Reyes, 2014). Imbalance between ROS and superoxide dismutase (SOD), glutathione peroxidases (GHS-Px) and catalases causes oxidative stress (Shah *et al.*, 2015). Metabolism of ethanol in liver produces reactive oxygen species (ROS) and reactive nitrogen species (RNS) which alter the biological process in liver. The process of inflammation enhances the alcoholic liver damage due to oxidative stress. The chronic ethanol consumption induces the oxidative stress, producing ROS and mitochondrial dysfunction (Galicia and Reyes. 2014; Shah *et al.*, 2016). There is always demand of antioxidants because our body is safeguarded by various natural antioxidants defense (Agbor and Nagogang., 2005). Hepatoprotective agents protect liver from hepatotoxic agents (Lee *et al.*, 2000). Synthetic and natural products are applied as hepatoprotective agents to cure the liver disorders. They protect liver or regenerate the hepatocytes (Pandey, 2012). Penicillin-G and Silymarin are approved antidotes used to treat liver diseases. When liver has drug-induced injury, N-acetylecysteline is used. Viral hepatitis has no specific drugs but interferons and nucleoside analog; lamivadine are widely used to treat chronic hepatitis (Mindikoglu *et al.*, 2006). Liver biopsy is suggested when autoantibodies are negative due to autoimmune hepatitis and prednisone is used to treat it (Stravitz *et al.*, 2011). When there is ascites, bacterial peritonitis and hepatic encephalopathy, then Norflaxacin and Trimethoprim antibiotics are used (Heidelbaugh and Bruderly, 2006). Chemically synthesized drugs have the developed adverse side effects and microbial resistance due to which man turned to ethnopharmacognosy (Duraipandiyan *et al.*, 2006). No appropriate drug can protect our liver from injuries. In spite of advancement in modern medicine, liver diseases are worldwide problem for health division. Hepatic cells play their role in variety of metabolic mechanisms. Therefore, there is a need to explore new liver protective agents against hepatotoxins, which may protect our liver (Russmann *et al.*, 2009). Herbal products have huge patronage worldwide as alternative to orthodox drugs (Mir *et al.*, 2010). Human health and welfare is immensely linked with medicinal plants (Cragg *et al.*, 1997). Human uses different parts of

plant in medicine but the potency of different plants to treat diseases is different which is correlated with their mechanism of action. Folklores herbs have plants extracts, which protect body from carcinogens (Bellini *et al.*, 2006). Plants have important chemical having multi-level and multi-targeted pharmacological activities and their efficacy have been explored (Ilyas *et al.*, 2016). World health organization (WHO) approves the plants or parts of plants, which are used for synthesis of drugs or healing of diseases, as medicinal plants (Ahmad *et al.*, 2011). Variety of plants-derived active compounds have potential to cure different diseases as well as liver disorders (Adeneye *et al.*, 2009). Even with the advancement in allopathic medicines, treatment of various diseases is carried out by medicines derived from plants (Sudipta *et al.*, 2012). Due to low toxicity and less chances of side effects, plants for treatment of different diseases since ancient times are practiced by human being (Elberry, 2011). Plants are an exemplary source of drugs due to which 80% people use plant based medicines for treatment of diseases (Vemula *et al.*, 2016). Plants based drugs are mainly used even their biological active constituents are not fully identified (Levy *et al.*, 2004). Recently, herbal medicines have gained popularity because of efficacy, safety and cost effectiveness. Medicinal plants are used as anticancer agents, antibacterial, anti-inflammatory, anti-viral and antioxidants in developing countries where hygienic facilities and health services are inadequate and infectious diseases are endemic (Rahim and khan, 2006). Natural drugs are less in number than the modern medicines to treat the diseases. Standarized plants extracts are going to be accepted in modern science for treatment of various diseases (Mir *et al.*, 2010). Potential active compounds of plants origin are used in drugs discovery (Lee *et al.*, 2012). Drugs discovery is a process to discover these potential active substances whereas the drug development is a process of identification of pharmaceutical drugs and to bring them into market. That is why there is need of sources for drugs discovery and drugs development for safe protection of body against different diseases including liver complication (Shuyi *et al.*, 2013).

1.2. Liver Disorder Management

Liver disorder management is big challenge for modern medicine and medical practioners (Bouasla *et al.*, 2014). Effective treatment of liver cirrhosis and chronic hepatitis with interferons, cholchicine, pennicillamine and corticosteroids are associated with serious side effects (Don and Rockey, 2005). No reliable and effective medicines are final that could prevent and treat liver diseases. Lot of research is focused to introduce hepatoprotective compounds from natural products (Valiathan, 1998). There is encouragement for frequent use of medicinal plants for hepatoprotective and hepatocurative (Gutierrez *et al.*, 2008; Wang *et al.*, 2010; Kumar *et al.*, 2013). Natural healing process of liver is accelerated with use of herbal drugs (Manjunath and Vidya, 2008; Patel and Shah, 2009). Well-documented traditional use of medicinal plants as hepatoprotective drugs is important one all over the world (Agarwal, 2001; Kumar *et al.*, 2013). Silymarin derived from plant i.e. milk thistle (*Silybum marianum*), shows promising activity to cure liver defects. *Phyllantus amarus* and Glycyrrhizin are used to treat chronic viral hepatitis. Some plants have proven significantly effective to treat liver disorder from China and Japan (Stickel and Schuppan, 2007). Silymarin is flavonolignan from *S. marianum*, is commonly used for liver protection in the field of health all over the world. It shows good protection in different experiments in laboratory animals with different toxins induced liver cirrhosis (Ghosh *et al.*, 2010). According to an estimate, out of 25000 to 500,000 species of plants, only 1-2% is well investigated for hepatoprotectivity. Efforts are made to get plant-based products to treat liver diseases (Mir *et al.*, 2010). From natural products, herbal extracts play an important role in recovery process of intoxicant livers. Hundreds of plants are reported to have been analyzed for wide range of liver diseases (Asadi-Samani*et al.*, 2013). Experimental assessment and formulations have clearly shown the efficacy of some medicinal plants to treat liver diseases (Kashawa *et al.*, 2011; Pandey, 2012). There is long history about treatment for liver diseases from natural therapy. Natural sources have anti-inflammatory, antioxidant, antiviral or antifibrotic and anticancer activities, but there is lack of standardization and limited toxicological evaluations (Thyagarijan, 2002; Mathur *et al.*, 2011). Antioxidants are reported to act against diseases by increasing endogeneous antioxidants levels and decreasing the

lipids peroxidation mechanism by scavenging action against free radicals (Bansal *et al.*, 2005). Medicinally important plants contain important phytochemicals like phenolic acid and flavonoids compounds that exhibit free radical scavengers due to antioxidant potential against carbon tetrachloride (Cheng and Ren, 2011). It is pre-investigated that hepatoprotective activity of plants extracts is due to their antioxidant properties (Laouar *et al.*, 2017). Compounds, which are metabolized by liver are toxic, so further research on hepatoprotective medicinal plants can trigger for safe evaluation for drugs discovery in their early phase (Adeneye *et al.*, 2009). There is growing attention on herbal medicines to treat liver diseases over the past decades. Remedies from plants are effective and safe alternative treatment for liver disorders (Yao *et al.*, 2016). Plants of valuable medicines may be screened to treat liver disorders because the herbal therapeutics are popularized world over by pharmaceuticals (Parma *et al.*, 2010; Chander *et al.*, 2014). Both invivo and invitro studies about flavonoids and phenolics of plants showed magnificient potency to prevent liver cirrhosis due to their strong antioxidant nature (Gebhardt, 2002).

1.3. Hepatoprotective Agents

Administration of hepatotoxic compounds into body elevates the serum biochemical components (ALT, AST and ALP) level in the blood. These are commonly known as biomarkers of hepatotoxicity (Uboh *et al.*, 2010; Saba *et al.*, 2012). More sensitive hepatotoxicity biomarker is ALT (Uboh *et al.*, 2010). Hepatotoxicity induction is commonly carried out by CCl_4 in various animal models (Alisi *et al.*, 2011; Ashoush *et al.*, 2013; Kumar *et al.*, 2013). When CCl_4 is induced in animal model then the level of enzymes such as ALT, AST, ALP and TB significantly increases (Laouar *et al.*, 2017). Administration of hepatoprotective or antioxidant efficient drug reduces the increased level of these enzymes in serum against CCl_4 intoxicant mice (Gbadegesin *et al.*, 2009). Conventional drugs are insufficient and have serious side effects while plants derived drugs are nontoxic and safe to use against liver disorders (Bhawna and Kumar, 2010; Mistry *et al.*, 2013). Plant based folk medicines are used to treat liver disorders, but there are not much drugs available that can be rely for effective treatment of liver complications (Vankat, 2015). There is need of urgent investigation of medicinal plant to study pharmacological effects of ethno-botanically

reported medicinal plants for liver treatment, which will be more helpful for liver protection and drugs development (Kumar *et al.*, 2012; Ashoush *et al.*, 2013).

Medicinal plants have been studies in various parts of Pakistan. It is need to have more investigations in order to promote herbal medicines (Virginia *et al.*,2012). There are many ethno-botanically reported medicinal plants used as folk medicines in Azad Kashmir, Pakistan. The north area of Azad Jammu and Kashmir (AJ&K) comprises high range of diversity and rich source of medicinal plants. As the local community of North region of AJ&K is highly dependent on medicinal plants for therapeutic purposes of different diseases, so there is need of urgent attention to study pharmacological validation of these plants for specific disease (Ishtiaq, 2015). Based on the ethnobotanical importance, some important indigeneous plants i.e *Geranium wallichianum*, *Thymus serphyllum*, *Viola canescens*, *Oxyria digyna*, *Elaeagnus parvifolia*, *Rosa bronunii*, *Dryopteris ramosa*, *Primula macrophylla*, *Aconogonon alpinium* and *Fragaria nubicola* (Qureshi *et al.*,2007; Qamar *et al.*, 2010 Adeel *et al.*, 2011; Khan, 2012; Bokhari *et al.*, 2013; Ch *et al.*, 2013) are selected from Poonch division of AJ&K, Pakistanto design a study about their hepatoprotective investigation. Literature survey shows that these plants have not been pharmacologically tested earlier so far against CCl_4 induced toxicity in mice for their hepatoprotective effects from Azad Jammu and Kashmir. Due to urgent need of safe hepatoprotective drugs and importance of these ethno-botanically important plants used for ailment of different helth problems, the aim of this study is focused mainly for investigation of hepatoprotective effect of these selected plants for pharmacological studies through in-vivo animal model against CCl_4 induced toxicity in mice so as to validate their efficacy for safe use for treatment of liver diseases.

1.4. Objectives of Current Study

Keeping in view the current prospective of hepatoprotective medicines and their necessity, my research is focused with following objectives.

1. Preparation of crude methanolic extract (ME) of selected plants of ethnomedicinal importantance.

2. Determination of hepatoprotective activity of crude methanolic extracts of selected plants and shortlisting of most active plants.

3. To determine the hepatoprotective effect and antioxidant activity of different fractions of short-listed most active plants to find out most active fractions.

4. Phytochemical analysis of methanolic extract and most active fractions of short-listed most active plants.

LITERATURE REVIEW

2. LITERATURE REVIEW

2.1. Structure and Functions of Liver

Liver comprising multiple lobules and sinusoids and the its portal vein brings 75% of blood supply to the heart from pancreas, spleen, stomach as well as from small intestine (Guyton *et al.*, 2006). Hepatic artery supplies only 25% blood to liver. Hepatic artery and portal vein empty themselves in sinusoids, which form vascular channel in the hepatic lobules. Sinusoids are site contents of hepatic artery and portal vein where they mix. Blood exits through liver by hepatic vein that merges to inferior vanacava to return the blood to heart (Hodgson, 2004). Functional cells of liver are hepatocytes, which form the liver lobules, endothelial cells and kupffer cells. Bile is also secreted by hepatocytes and capsule of connective tissue surrounds the whole liver and makes division of lobes into lobules (Smith, 2005). Liver is involved in metabolic functions of body (Kumar *et al.*, 2012). Liver performs various metabolic activities such as; amino acids synthesis, carbohydrates and lipids metabolism, production of coagulants (fibrinogen, prothrumbin etc) and bile secretion for emulsification of fats. Liver plays its activities in breakdown of toxic metabolites (drugs and toxins) and converts ammonia into urea through urea cycle. Storage of various vitamins like vitamin D, vitamin A, vitamin B12 and vitamin K, copper and iron takes place in it (Ramadori *et al.*, 2008). Liver plays its promising function of metabolism and to excrete metabolites, generated in the body from drugs and xenobiotics. Liver disorder is huge challenge for pharmaceutical industry and healthcare professionals all over the world (Heidelbaugh and Bruderly,2006).

2.2. Liver Defects and Causative Agents

Liver damages are associated with the change in metabolic activities. Habitual consumption of alcohol, exposure to drugs interaction and xenobiotic lead to liver dysfunction (Kumar *et al.*, 2013). Liver disorder is one of the defects, which lead to hepatitis, Jaundice, cirrhosis and liver carcinoma. Many drugs such as chloroquine and isoniazide

induce toxicity and chronic liver diseases. Liver fibrosis degrades the hepatocytes. There is report that in 2001, about 2700 deaths in United States occurred due to cirrhosis of liver and this range is 10th in males and 12th in females (Jacob *et al.*, 2014). Long term ingestion of chemicals from different resources and environment are agents reported to cause health problems which lead to hepatitis, cirrhosis and liver damages leading to fatal in the world. Thousands of synthetic chemicals, drugs, bacteria, fungi, plants and animal toxicants cause hepatotoxicity (Raju, 2008; Heibatollah, 2008). In addition to this, environmental pollutants and different hepatotoxic chemicals cause the hepatic injury, which produce different types of liver diseases. Other factors which play their role to accelerate liver injury are oxidative stress and pathophysiological roles of free radicals that lead to damage liver permanently (Chandan, 2008; Feijoo, 2010). Due to lack of proper mechanisms for handling sewage, disinfection, poor supply of clean water; all sorts of hepatitis are prevalent which cause liver diseases in Pakistan (Butt, 2015).

2.3. Mechanisms of Liver Toxicity

Liver injuries are damages of hepatic parenchyma and are associated with liver metabolic distortion (Stickel and Schuppan, 2007; Law and Brunt, 2010). Free radicals are unstable chemical compounds having unpaired electrons in the outermost orbit. They always need the other electron for pairing with and become stable. They interact the biological molecules like proteins, carbohydrates lipids and nucleic acids (DNA & RNA) in the body and steel the electrons which results the damage of these biomolecules which initiates uncontrolled reactions and hence production of these free radicals resulting the diseases like liver problems also (Valko *et al.*, 2006). Reactive oxygen species (ROS) are important free radicals which have two sources, endogenous sources and exogenous sources which are produced in mitochondria during production of energy (Bergendi *et al.*, 1999) and group of enzymes called cytochrome P450 mixed function oxidase in the liver which use molecular oxygen (Lieber, 1996). Smoking, toxic chemicals, radiations, air pollutants, pesticides and organic solvents that come from environment are exogeneous sources (Buyukokuroglua *et al.*, 2001). Increased level of free radicals in the liver, cause the damage during decreased scavenging potential of cell (Natarajan, 2006). When hepatocytes are damaged, bile acid addition takes place that accelerates the liver damages (Pingale, 2010). When hepatic cellular

damages take place then immunological dysfunctions also prolong. Stress and damages cause the signals to release which activate new cells. Kupffer cells (K) and Natural killer cells (NKC) which are largest population of innate cells of liver; produce pro-inflammatory mediators and secretions of chemokines take place. Inflammatory cytokines, interleukins and tumor necrosis factors (TNF) are produced due to hepatic injury and they further promote the liver tissue damage by suppressing the hepatoprotective immune cells by mechanism of stress (Bilzer *et al.*, 2006; Kashawa *et al.*, 2011).

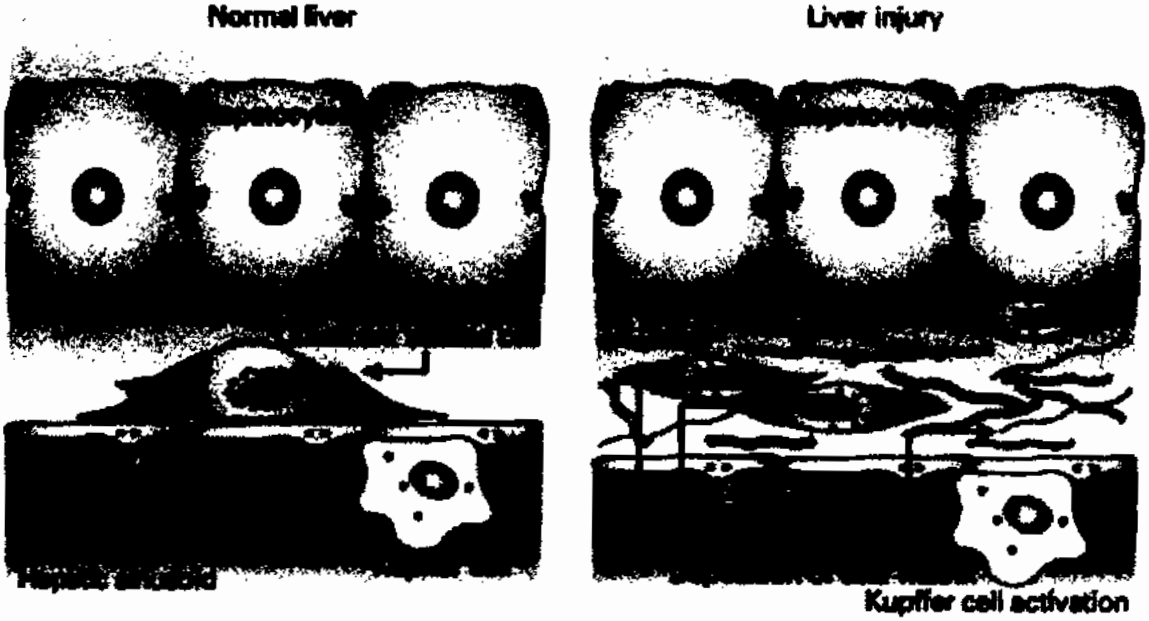


Figure 2.1: Illustration of Four Types of Liver Cell (Hepatocytes, Kupffer Cells, Endothellial Cells and Stellate Cells) in Healthy and Injured Liver (Asselah *et al.*, 2009).

2.4. Hepatitis; an Alarming Factor to Damage Liver

Viral hepatitis damages the liver. Dark urine and jaundice symptoms in patients of hepatitis are reported which show that liver infections lead to liver failure and ultimately patients get die. There are around 1.5 million clinical cases of hepatitis A (HAV) all over the world, which causes the liver damage and deaths. In developing countries liver infections in children are about 50% while in adults this range is 75% that develops jaundice (Franco *et al.*, 2012). There are around 350 million people chronically infected with HBV (Trans *et al.*, 2006). A study shows that frequency of different cases that lead to liver cirrhosis in local

population of Pakistan are, hepatitis B (18%) and hepatitis C (61%). WHO's reports show that after Egypt, Pakistan is at second position in prevalence of HCV. Chronic HCV infection causes liver damage, which leads to cirrhosis. Co-infection of Hepatitis D and hepatitis B increase the rate of liver cirrhosis (Friedman, 2014). There are about 20%-30% patients with chronic hepatitis that develops cirrhosis. HCV and alcoholic liver damages are common reasons for liver transplant (Longo, 2011). Worldwide estimation of chronic hepatitis infection is 71 million. In Pakistan, the leading factors of liver diseases is hepatitis C that spreads due to unsafe injections and unhygienic instruments (WHO, 2015). Alcoholic liver diseases are about 20% in US and in 2003; the cases reported were 44%. It is 10^{th} reportable disease in Pakistan (Ullah *et al.*, 2012). Pakistan has a significant number of people with liver disorders having fatigue, difficulty in sleeping, muscles cramps, dryness of mouth and itching (Atiq *et al.*, 2004). Alcoholism and xenobiotics are other chemicals that injure the liver and this secondary liver damage is reported to be the side effect of deferasirox, used to treat Haemochromatosis (Naeem *et al.*, 2010).

2.5. Treatment Methods of Diseases

Ancient therapeutic practice in the world is complementary and alternative medicines (CAM). Stringent research protocol is followed to produce manufactured drugs, which enable to reduce substantial side/toxic effects. Plants in industrialized and developing countries are still practiced by populations. Those populations consider the medicinal plants as useful therapeutic source and are motivated by believes that these products are naturally obtained and have less side effects than synthesized drugs that are practiced conventionally (Bruneton, 1993). Treatment of multiple diseases through synthetic drugs is associated with undesirable hazards and side effects. There is increasing shift from synthetic drugs to CAM especially through plants extracts because they are accepted culturally, available easily and economically viable (Kumar *et al.*, 2013). About 80% of populations in developing countries treat the health problems through medicinal plants (Nagpal and Sood, 2013).

2.6. Role of Plants in Healthcare

Medicinal plants having major source of medicines for pharmaceutical industries in the world that is why new pharmaceutical drugs are obtained from plants. There is a fact that wildly growing plants have not been much studied for their pharmaceutical potentials. The evidences of herbal medicines are about 5000 years old. Egyptian papyri described various beneficial herbs (Aboelsoud, 2010). About 224 drugs which are mentioned in Huangdi Neijg (Chinese medical treatment) are herbs (Hong and Francis, 2004). Since allopathic medicines are quite expensive for common people, therefore, they rely on herbal medicines from nature. Many compounds derived from plants are used in development of modern medicines on pharmacological based tested evidences. Raw material of these potentially beneficial plants are also supplied to herbal industries for drug development (Bhattarai *et al.*, 2008). Various studies have shown that antibiotics, anti-inflammatory and antioxidant agents are of plants origin (Rahman *et al.*, 2012; Bokhari *et al.*, 2013). Diagnosis, treatment and care of various diseases are attributed to potential application of pharmaceutical drugs (Robson and Vaithiligam, 2009). Aspirin, opium and quinine, which are plants derivative drugs are prescribed by many physicians (Fabricant and Norman, 2011). World Health Organization (WHO) has estimated that about 80% populations rely on herbal drugs to treat different diseases in Asia and Africa. Many valuable drugs for cancer therapy (podophyllotoxin, taxol, vincristin and camptothecin) derivatives of plants (Komalavalli *et al.*, 2014). Plants metabolites have common potential applications like radical scavengers and metal chelaters (Nithya and Balakrishnan, 2011). About 25% drugs are reported to be plants derived in America and 74% of 119 drugs are important, whose ingredients are derived from traditional medicines. *Elephantopus scaber* (L) is known to be used as traditional remedy by Chinese to reduce fever, stimulate diuretic and eliminate bladder stones as well as to treat nephritis, chest pain, edema, pneumonia, scabies and leukemia. A number of phytochemicals have been isolated from this plant (Wan *et al.*, 2009). Pharmaceutical research also focuses on natural products to get new active molecules (Vaishali *et al.*, 2013). There are at least 120 active compounds that have been isolated from higher plants. Modern medicines and traditional usage of herbs shows 80% positive correlation of these active compounds (Cravotto *et al.*, 2010). People in Thailand use medicinal plants to treat different diseases such as digestive

disorders (Tangjitman*et al.*, 2015). Various factors such as environmental pollution, overpopulation, urbanization and industrialization are common threats of toxicity faced by Pakistan. Food chain system is found contaminated with heavy metals toxicity and hydrocarbons (Shinwari, 2010). Folk people in different regions of the world use plants for medicines. In Pakistan herbal medicines are not prescribed by doctors due to lack of information about them. They have suspense about toxic effect of active compounds for patients (Husain *et al.*, 2011). Himalayan region people depend on plants for diseases cure (Shaheen *et al.*, 2014; Shaheen *et al.*, 2017). Northern areas of Pakistan are rich in *Indigofera heterantha* which have number of medicinal uses such as traditional remedy for spastic pain, abdominal pain and to treat problems of skin (Ghiasuddin *et al.*, 2011). Drug resistant pathogens are reported to treat by seventeen medicinal plants used as alternative (Malik *et al.*, 2010). According to study, various diseases such as circulatory problems, stomach disorders and urinary problems are treated with 101 medicinal plants by Western Himalayan region people of Pakistan (Khan *et al.*, 2012).

Azad Jammu and Kashmir, Pakistan has rich flora due to diversified habitats such as springs, rivers, meadows, steep mountains slopes and lakes. From Kotli Azad Kashmir, the plants recorded are mostly medicinal and they belong to family Rhamnaceae (Ajaib *et al.*, 2010). About 50 species of medicinal plants that are climbers/runners have multiple medicinal applications (Ajaib *et al.*, 2012). Ethno-botanical study from Bagh Azad Kashmir Pakistan, gives therapeutic knowledge about 33 medicinal plants species of angiosperms plant. Diseases are treated using single plant or in combination of different herbs. Diseases such as diarrhea, liver damages, cough, malaria, and stomach disorders are effectively cured by these plants (Qureshi *et al.*, 2007). Drugs from plants can be easily achieved because the plants are treasure house of medicines. Bioactivity screening and toxicity test provides the efficacy and suitability of these products from medicinal plants (Wan *et al.*, 2009). *Rheum emodi* is an important Himalayan herb that has medicinal value. There are about 60 perennial species of *R. emodi* distributed on the world. It has nephroprotective, antimicrobial, anti-inflammatory, anti-cancer and anti-oxidant activity (Rehman *et al.*, 2014). *Polygonum minus* has close relationship between traditional and modern sources for ethno-pharmacological uses, especially for treatment of ulcer, cytotoxicity, hepatic abnormalities and protozoa

infections (Ganapathi and Jamaludin, 2014). Leaves and roots of *Clitoria ternatea* are useful to treat number of diseases such as stings of animals, urinogenital disorders and aches (Patil and Patil, 2011). This plant has anti-inflammatory, blood platelets inhibitory, anti-diabetic, diuretic, analgesic and smooth muscles relax pharmacological properties (Bhawna and Kumar, 2010).

2.7 Hepatoprotective Role of Medicinal Plants

Many medicinal plants are hepatoprotectice (Heibatollah*et al.*, 2008). Folk medicines from plants origin have been reported as potential remedies against the liver injuries (Vankat *et al.*, 2015). There are several reports from Asia, Europe and America where medicinal plants are practiced by most populations to treat liver diseases (Gutierrez, 2008; Kumar *et al.*, 2013).

The key testing procedure of medicinal plants for their pharmacological activity is presented by invivo models i.e rodents' animal model. Hepatoprotective effect of extract are tested invivo using hepatotoxic compounds such as ethanol, paracetamol, CCl_4, caffeine and cadmium (Mukazayire *et al.*, 2010; Osadebe *et al.*, 2012). Degree of protection/toxicity of substance are known by measurements of liver markers (ALT, AST, and ALP) after administration of testing compounds (Chandan *et al.*, 2007). Integrity of liver is assessed by histlogical studies and biochemical assessment of change in levels of biomarkers of liver (Uboh *et al.*, 2010). When carbon tetrachloride is administered for hepatotoxicity, leakage of liver markers i.e. ALT, AST and ALP from hepatocytes takes place by membranes damage leading to significant effects in blood serum which justifies biochemical markers for the hepatotoxicity. Administration of known hepatoprotective or antioxidant efficient drug in mice reduces the concentration of these enzymes in the serum and provides a scientific proof that this extract is hepatoprotective (Nada, 2010; Saba, 2012).

Clitoria ternatea is a medicinal plant in tropical region of equatorial Asia and its hepatoprotective activity has been investigated experimentally (Nithianantham *et al.*, 2011). Investigation shows that when albino rats were induced by hepatotoxin (acetaminophen) and leaf extract of *Alchornea Cordifolia* (medicinal plant), it showed liver recovery from

damages (Jacob *et al.*, 2014). Pharmacologically few plants efficacy have been evaluated to treat liver damages. Chemical constituents from plants of family Lamiaceae have been studied and their biological roles with concentrations have been correlated (Elhardallou, 2011). Phytochemical such as terpenoids, carbohydrates and flavonoids have been extracted from ethanol extract of *Ocimum canum*. Similarly, there is also report of presence of tannins, phenols, glycosoids, saponins and steroids from chloroform extract of *Ocimum canum* leaves that are hepatoprotective (Vaishali *et al.*, 2013). The medicinal plants; *Prospermum acerifolium*, *Cleome viscose*, *Andrographis lineate*, *Phyllanthus reticulatis*, Andrographis *puniculata*, *Morinda citrifolia*, *Eclipta alba*, *Swertia chirata* and *Wedelia calendulacea* have been reported to be used for treatment against CCl_4 induced toxicity in animals. They have potential effects against the liver disorders. Similarly, there are other plants, which have been reported hepatoprotective against rifampicin, and acetamenophene (paracetamol) treated toxicity. These plants include *Casia fistula*, *Solanum nigrum*, *Careya arborea*, *Fumaria indica* and *Azadirachta indica* (Bhawna and Kumar, 2010). *Elytraria acaulis* extract applied on mice against CCl_4 induced toxicity showed hepatoprotective effects. The decline in ALT, AST and TB in rats, treated with *E. acaulis* extract were the indications of the hepatoprotective activity of *Elytraria acaulis* (Reddy *et al.*, 2014). Ethanolic extract of*Elytraria acaulis*shows the hepatoprotection similar to hepatoprotective results of polyherbal formulations against liver damages induced by CCl_4 treatment (Vilas *et al.*, 2010). The histopathological study of liver showed that 90% ethanolic extract of *Evolvulus alsinoids* normalized the architecture of liver as compare to silymarin. *Evolvulus alsinoids* extract contains flavonoids which posess the hepatoprotective activity (Chander *et al.*, 2014). Out of 52 ethno-botanical uses of plants, 21 plants have been reported from Taripura state of India, which show hepatoprotective role (Sudipta *et al.*,2012). After the induction of liver damage by acetaminophen, the significant increase of serum liver markers i.e. ALT, AST and ALP levels and decrease in level of total proteins (TP) shows that extract of *Cichorium intybus* posess potential hepatoprotective role (Butt *et al.*, 2012). Plants including *Picrorrhiza kurroa*, *Curcuma longa*, *Glycyrrhiza glabra*, *Camellia sinensis*, *Silybum marianum*, *Bupleurum falcatum* and *Taraxacum officinale* have potentioal to treat liver disorders reported from China, India and Europe (Mukazayire *et al.*, 2010). Ethanolic extract of

Zenthoxylem armatum against carbon tetrachloride (CCl_4) induced toxicity proved to decrease the liver serum bilirubin, ALP, ALT and AST (Verma and Khosa, 2010). Similar study on hepatoprotective activity of *Rumexdentatus* against paracetamol intoxication in mice showed the significant decrease in AST, ALT and total bilirubin (Saleem *et al.*, 2014). Carbon tetrachloride (CCl_4) induced toxicity is also reported to be recovered by polyherbal extracts that are proved strongly hepatoprotective (Vilas *et al.*, 2010). Hepatoprotective potential of*Rheum emodi* have been proved by treating rats with CCl_4. Oral administration of*R. emodi* extract, about 300mg/kg significantly decreased the increased liver parameters. Similar hepatoprotective activities of aqueous extract of *R. emodi* against liver damage induced by paracetamol in albino rats have been confirmed (Ibrahim *et al.*, 2008; Akhtar *et al.*, 2009). *R. emodi* has ability to treat liver jaundice and is cultivated in different countries of the world (Tahir *et al.*, 2008). Traditional medicines from plants are obtained in different combinations as liver tonic in Iran. These are used to treat liver disorders (Asadi-Samani *et al.*, 2015). Experiments show that lipid per oxidation level was restored towards normal values by treatment with extracts of *Phyllanthus niruri* and *Maytenus emarginata* against paracetamol induced toxicity in rats (Parma *et al.*, 2010). Ailment of liver diseases like Hepatitis, Cirrhosis and loss of appetite is carried by plant medicines. Some important plants are *Cassia alata*, *kampferia rotunda* and *Keampferia galangal*. In another study, 21 more plants which have hepatoprotective effects, have been recorded (Sudipta *et al.*, 2012). Silymarin had been used as raw extract from seeds of *Silybium marianum* that showed hepatoprotective effects against isoniazid induced toxicity in rabbits. It can be used for therapy in patients having hepatic problems (Sana *et al.*, 2010).

2.8. Phytochemicals as an Antioxidant

Chemical substances that have plant's origin and cause any physiological action in the human body are called phytochemicals. Herbal and homeopathic medicines use these phytochemicals since the ancient time to treat different diseases. These have diseases preventive or curative property but no nutritive in nature (Genene and Hazare, 2017). Different metabolites of plants such as saponins, flavonoids and tannins from *Conyza sumatrensis* are reported (Shah *et al.*, 2012). Medicinal plants investigated for phyto-

constituents showed presence of secondary metabolites such as tannins, saponins, phlobatanins, terpenoids, steroids, glycosides and flavonoids (Njoku and Obi, 2009). An antioxidant activity of two diterpenoids has been reported that have been isolated from *Cladonia rangiferina* (Yoshikawa*et al.*, 2008). Plant barks, leaves, roots, flowers and fruits are important parts from which drugs can be isolated. Screening of total flavonoids and phenolic from seven medicinal plants of different families at India have been reported. These plants included *Oldenlandia corymbosa*, *Xanthium strumarium*, *Ricinnus communis*, *Terminalia bellirica*, *Tinospora cordifolia*, *Ipomea aquatica* and *Bryophyllum pinnatum* (Yadav and Agarwala, 2011). Phytoconstituents prevent hepatic diseases by stimulating the immune system (Ilyas *et al.*, 2016). Silymarin, β-sistosterol, neoandrographolide, phyllanthin, curcumin, picroside, betalain, andrographolide, hypophyllanthin, glycyrrihizin and kutkoside are reported for their potential hepatoprotective properties that are derivaties of medicinal plants (Asadi-samani *et al.*, 2015). Phytochemical composition of *Bauhiniahookeri* including gallic acid, trimericprocyanidins, hydroxycinnamic acid derivatives, flavonoids and dimeric have hepatoprotective effects (Sayed *et al.*, 2014). Phytochemicals play an important role in plants for their defense (Vaishali *et al.*, 2013). Many natural compounds isolated from plants having free radical scavenging potential are considered therapeutic agents for liver damages. Tannins; high molecular weight polyphenols are also found naturally in medicinal herbs and have a major role in free radical scavenging activities to treat liver diseases (Rehan *et al.*, 2014). Synthetic antioxidants i.e. Vitamin C, vitamin E and beta-carotene available in market caused mortality in adults who used them. This mechanism is due to their toxicity as compared to natural antioxidants (Bjelakovic, 2007). Hepatoprotective abilities of plants are due to antioxidant potential and free radical scavenging property of their constituents (Nithianantham *et al.*, 2011). It is also known that mixtures of antioxidant compounds are more active than the individual components (Prochazkova *et al.*, 2011). Antioxidant activity of leaves of different varities of sweet potato is reported for correlation with phenolic and flavonoids contents, which concluded that Ipomoea potatoes leaves are sources of natural antioxidants that possess radical scavenging activity to free radical DPPH (Hue, 2012). The relationship between the high level of phenolics (e.g. phenolic acid) and radical scavenging activity have been reported

by several studies on medicinal plants (Alonso *et al.*, 2004; Bertoncelj *et al.*, 2007; Céspedes *et al.*, 2008; Park*et al.*,2008). Antioxidant potential of some medicinal plants has also been studied i.e. *Cichorium intybus* has richest source of natural antioxidants (Rafique *et al.*, 2014). Antioxidant phytochemicals play potential hepatoprotective role against paracetamol-induced toxicicity in mice (Gyawali *et al.*, 2017). Potential of *Pomegranate* peel and Whey powder have been evaluated for CCl_4 induced toxicity for its antioxidant activities and hepatoprotective effect. The extract proved its antioxidant and hepatoprotective activity significantly (Ashoush *et al.*, 2013). Natural antioxidants derived from plants are alternative to synthetic medicines that protect from free radicals in body. Maintenance of health and diseases prevention is correlated to diet enriched with fruits and vegetables with the development of interest to identify more natural antioxidants (Pagang*aet al.*, 1999; Scalbert and Williamson, 2000). Flavonoids play biological effect like scavenging of free redicals and prevent cell proliferation and fight oxidative stress. Many flavonoids have exhibited anti-oxidative activity, free radical scavenging, hepatoprotective and anti-inflammatory effects (Kumar and Pandey, 2013).

Typical phenolic acid includes caffeic acid, ferulic acid and vannilic acid that are important natural antioxidants. The diverse phenolics are flavonoids widely distributed in plant kingdom. Flavnol glycones (include quercetin, myriectin, campferol and glycoside) are constituents of flavonoids and their antioxidant activity is due to multiple hydroxyl groups. Redox property of antioxidants enables them to act as reducing agents (Exarchou *et al.*, 2002; Darwisd *et al.*, 2008). There is literature report about studies of total phenolic contents (TPC) and antioxidant activity of medicinal herbs used in traditional remedies. There is positive correlation between total antioxidant activity and total phenolic contentents in which O*riganum vulgar* extracthas highest antioxidant ability (Spiridon, 2011). When synthetic drugs are applied to treat different disorders, there is generation of free redicals which cause other side effects and diseases. Phytoconstituents are compitable to body physiology and prevent free radical reaction. Studies about free radical scavenging potential of *Cassia auriculata* extract showed that it has potential source of antioxidant in acetone extract of leaves, fruits and stem. This can be applied as antioxidant source in modern era of medicine (Gaikwad *et al.*, 2011). Large number of medicinal plants and their constituents are reported

for their antioxidant activity that possess isoflavones, flavonoids, anthocyanin, cumarin, lignins, catechin and isocatechin in which green tea has highest scavenging effects to free radicals.These plants include *Camellia sinensis*, *Ocimum sanctum*, *piper cubeba* Lin,*Allium sativum*, *Terminallia bellerica*, *Zhingiber officinale* (of Middle East, Palestine and India) and several Chines and Indian plants where several diseases are treated by antioxidant-formulated drugs (Aqil *et al.*, 2006; Nooman *et al.*, 2008).

2.9. Silymarin as an Important Standard Hepatoprotective Drug

Molecular Formula: $C_{25}H_{22}O_{10}$

Molar Mass: 482.44g/mol

Silymarin is mixture of 3 flavonolignans isomers which are silybin, silydianin and silychristin in which silybin is most active component of Silymarin (Kvasnicka *et al.*, 2003). Silymarin is isolated from seeds of milk thistle (*Silybum marianum*). Its protective mechanism is by binding with hepatotoxin binding sites to cell of hepatocytes membrane. It has antioxidant, anti-inflamtory, hepatoprotective, redicals scavenging properties, antifibrotic effects, enhancement of hepatocytes regeneration and reduction of glutathione. It is orally absorbed but poor water solubility (Kshirsagar, 2009). Various researchers demonstrated hepatoprotective activity of silymarin using animal model against thioacetamide (TAA), paracetamol, CCl_4 and ethanol. It has better patient tolerability, good safety profile, therapeutic efficacy and cost effective (Pradhan and Girish, 2006; Dixit *et al.*, 2007; Ghosh *et al.*, 2010). A study report shows that there is strong hepatoprotective effects against the CCl_4 intoxicant mice with ethanolic extract as compare to ethyacetae extract and these results are closely related to hepatoprotective agent; Hepaticum (Shaker *et al.*, 2010). Other reference compounds which play important role in the hepatoprotective mechanism against toxicants are vitamin A, C and E but the silymarin is most important one and plays its protective role to liver diseases due to its antioxidant properties by supperesing the oxidative stress (Raza *et al.*, 2011). GSH is also hepatocytes protective compound which is tripeptide synthesized by mammalian cell and also by the hepatocytes of liver due to which

it is also used as reference hepatoprotective agent in clinical trials especially against paracetamol induced toxicity (Albano *et al.*, 1985).

Establishment of efficacy for safe use of all herbal drugs is desireable (Qureshi *et al.*, 2007). The plants species i.e. *Podophyllumhexandrum* Royle, *Berberis lycium* Royle, *Oxyria digyna* (L.) Hill, *Rheum emodi, Rheum austrail*, *Aconogononalpinum*, *Angelica cyclocarpa*, *Solanum nigrum*, *Arnebia benthamii*, *Geranium wallichianum*, *Viola canescens*, *Morchell esculenta*, *Jurinea himalaica*, *Rhus succedanea* and *Saussurea lappa* are important for medicinal values from Neelum Valley Azad Kashmir Azad Jammu and Kashmir, Pakistan (Adeel *et al.*, 2011). Hepatoprotective effects about these plants are little known.

Carbon tetrachloride (CCl_4) is well known for its hepatotoxic effects. CCl_4 is useful in laboratories to induce acute-toxic liver injury in animals (Ashoush *et al.*, 2013). Due to importance of medicinal plants as key source of hepatoprotective drugs and increasing cases of liver damages, a study has been focused for screening of hepatoprotective effects of methanolic extracts of some medicinal plants from different localities of Poonch division of Azad Kashmir, Pakistan against CCl_4 induced toxicity in mice. These plants are *Geranium wallichianum*, *Thymus serphyllum*, *Viola canescens*, *Oxyria digyna*, *Elaeagnus parvifolia*, *Rosa brunonii*, *Dryopteris ramosa*, *Primula macrophylla*, *Aconogonon alpinium* and *Fragaria nubicola.*

2.10. Description of Plants Under Study

2.10.1. *Viola canescens*

Family: Violaceae **Varnacular Name:** Thundi-jari /Banafsha

Status: Wild herb **Flowering period:** March-July

Distribution: Ethnobotanical study shows its distribution in different localities. It is also called Himalayan white violet. It is reported to present in Nepal, India, Pakistan and Bhutan. It is widely distributed in Neelum valley, Bagh, Poonch, Haveli and Muzafarabad Azad Jammu and Kashmir.

Ethno-medicinal Uses: Whole plant extract is used for liver problems. Leaves, roots and flowers as vegetables use. Malaria, fever, bronchitis, liver and gastric problem are treated by common people of Neelum AJ&K (Adeel *et al.*, 2011). It is used for pain relive and traditional medicines (Abbasi *et al.*, 2013).

Previous Studies: There is also report about anti-parasitic, gastrointestinal and analgesic effects on mice (Maria *et al.*, 2014).

Figure 2.2: *Viola canescenes* with Flowers

2.10.2. *Dryopteris ramosa*

Family: Dryopteridaceae Vernacular name: Pakha/Kunji

Status: Wild herb Flowering period:Non-flowering plant

Ethno-medicinal Uses: It is used for digestive disorders. Its leaves are used as fodder. Juice is used for treating stomach pain (Ahmad and Habib, 2014).

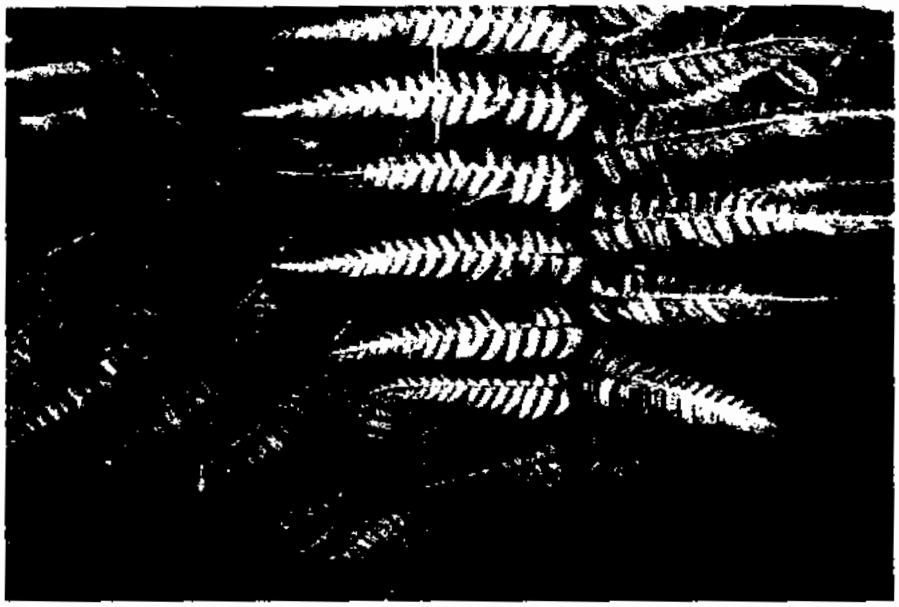


Figure 2.3: *Droyopteris ramosa* Shoots and Leaves

2.10.3. *Geranium wallichianum*

Family: Geraniaceae **Vernacular name:** Rattenjot

Status: Wild herb **Flowering period:** June-September

Distribution: It is distributed in Himalayas from Kashmir to Nepal at altitude of 7000-11000 feet. It is present at different localities of Northern areas, Hazara, Azad Kashmir and Murree hills, Pakistan (Ahmad*et al.*, 2003).

Ethno-medicinal Uses: Its leaves and floral parts are used to treat eye problems and for purification of blood. Jaundice, spleen problems and kidney disorders are treated by its root powder (Qureshi *et al.*, 2007). It is also used to treat Hepatitis, old fever, premature delivery and leucorrhoea (Adeel *et al.*, 2011).

Previous Studies: *Geranium wallichianum* is reported to study about its biological activities. Six compounds (β-sitosterol, ursolic acid, stigma sterol, sistosterol, galactoside, herniarian and trioxyethyl benzoate) are reported to contain in it having antioxidant role for which ethyl

acetate is highly potent having IC_{50} 19.05 µg/mL than n-butanol and aqueous extracts (Ismail *et al.*, 2009). A review study shows that another species, *Geranium robertianum* has phytochemical characterization of phenolic compounds especially for flavonoids about their anti-oxidant, anti-inflammatory and anti-hyperglycemic effects (Vania *et al.*, 2016). Antimicrobial, cytotoxic, antifungal, enzymes inhibitory activities and insecticidal studies are also reported (Ismail *et al.*, 2012). There is no record found about its hepatoprotective activity in animal model using carbon tetrachloride as hepatotoxin from Azad Jammu and Kashmir (AJ&K).

Figure 2.4: *Geranium wallichianum* with Flower

2.10.4. *Elaeagnus parvifolia*

Family: Elaeagnaceae **Vernacular name:** Kankoli

Status: Woody wild shrub **Flowering period:** May-August

Ethno-medicinal Uses: It is cardiac stimulant. Its seeds are used against cancer. Leaf infusion is used as diuretic. People of AJ&K, Pakistan use it as traditional practices to form juice and jams (Khan *et al.*, 2010). Its fruits are juicy and pleasant. It is used to treat

pulmonary infection and cough by inhabitants of Azad Kashmir, Pakistan (Amjad *et al.*, 2015).

Previous Studies: According to literature search, there is no record of pharmacological studies of *E. porvifoila*. However, record of pharmacological studies is reported about other species of *Elaeagnus* (Liao *et al.*, 2012).

Fig 2.5: *Elaegnus parvifolia* with Fruits

2.10.5. *Rosa brunonii*

Family: Rosaceae **Vernecular name:** Tarnari/ chahal

Status: Wild Shrub **Flowering period:** May-July

Ethno-botabical Uses: It has medicinal and ornamental uses. Powder of flowers is used for skin diseases while extract of roots to treat eczema (Khan *et al.*, 2012; Ahmad *et al.*, 2012a).

Previous Studies: It is reported to present in Muzaffarabad Machiara Park, Bagh, Neelum and Poonch Azad Jammu and Kashmir, Pakistan (Dar *et al.*, 2012).

Figure 2.6: ***Rosa brunonni*** **with Flowers**

2.10.6. ***Aconogonon alpinum.*** **Schur**

Family:	Pologonaceae	**Vernacular Name:**	Masloon/ Chukroo
Status:	Wild herb	**Flowering period:**	June-September

It is perennial herb distributed at varios zones like dry meadows, forests glades, stoney slopes, alpine zone and cliffs. Distributed in, Mangolia, China, Japan and former USSR. Asia and Westeren regions of North America, there are reports about 35 species of this genus (Malyshev and Peshkova, 1979; Yasmin, 2015). It grows to 2000-4000 m in open meadows and shady slopes. It has been found to distributed in North Western Himalyas and Siberia. There is record of five species of this genus in Pakistan (Qaiser, 2001; Yasmin, 2015). In Azad Jammu and Kashmir, Pakistan it is present at alpine regions of high mountainous forests (Qureshi *et al.*, 2007; Ch *et al.*, 2013).

Ethno-medicinal Uses: The seeds are used as purgative and emetic by local people while fever and menstruation are treated from its floral parts (Ch *et al.*, 2013). Stomach disorders

are treated by this plant and its roots are carminative (Adeel *et al.*, 2011). Liver Jaundice in North region of Azad Jammu and Kashmir is treated by folk people by its roots also.

Previous Studies: Flavonol complex is reported from Altay. Phytoconstituents like astragalin, glycosides, quercetrin, hyperoside, avicularin, rutin, quercetin 3,7-diglucoside, myricitrin, and aglycones (quercetin, kaempferol and myricetin) from above-ground parts of this plant are reported. The chemical contents are reported to investigated through HPLC in which high content of flavonol is about 10.35% and has low toxicity and high biological activities (Vysochina and Khramova,2010). There is no report about work on pharmacological investigation especially about hepatoprotective effects against hepatotoxin induced damages in animal models.

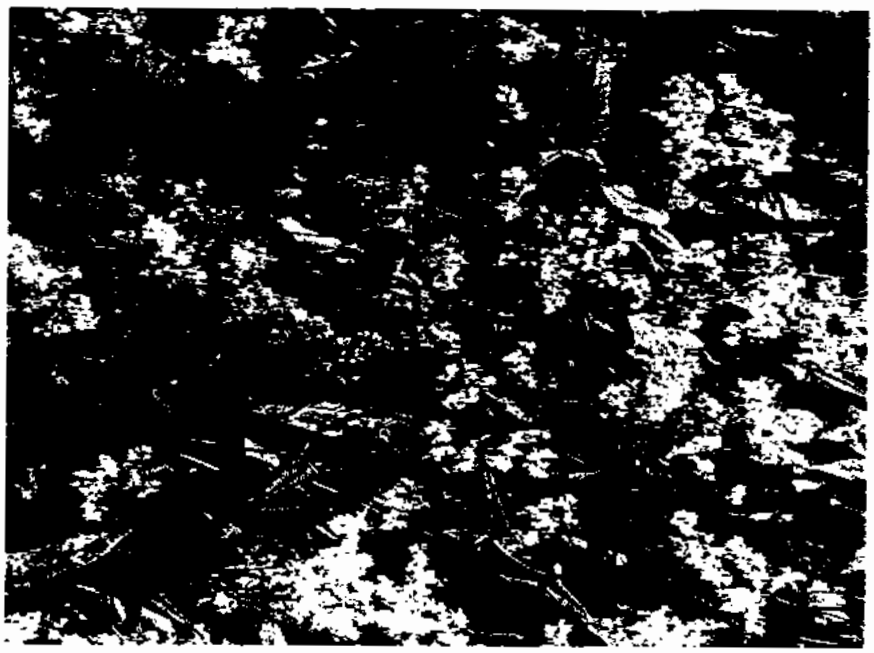


Figure 2.7: ***Acconogonon alpinum*** **with Flowers**

2.10.7. *Oxyria digyna*

Family:	Polygonaceae	**Vernacular name:**	Khutery
Status:	Wild herb	**Flowering period:**	June-August

It is also reported to distribute in Hilamiyas region. In Neelum Valley AJ&K, it is considered as an importantmedicinal plant. Ethno-medicinal uses: Mostly shoots are used to extract juice that is used for constipation; liver disorder and stomach ache (Adeel *et al.*, 2011). It is also used against jaundice and thrust (Husain*et al.*, 2011).

Previous Studies: Free radical scavenging properties and phenolic characterization showed that Vitexin is present in *Oxyria digyna* which has best antioxidant potential (Orhan *et al.*, 2009).

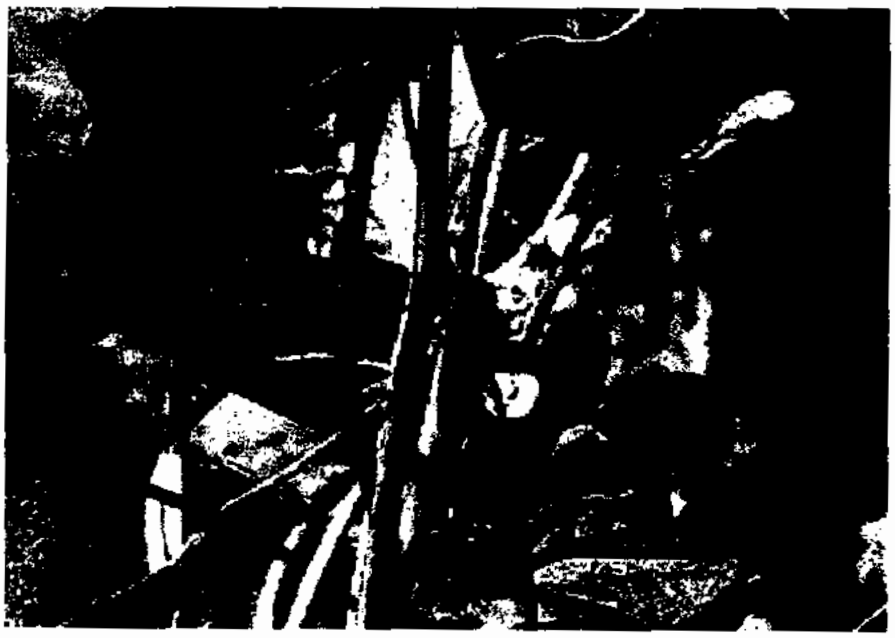


Figure 2.8: *Oxyria digyna* Shoot with Flowers

2.10.8. *Fragaria nubicola*

Family:	Roseaceae.	**Vernacular Name:**	Budmewa
Status:	Wild herb	**Flowering period:**	May-July

Distribution: Distributed in Bagh and Poonch region of AJ&K Pakistan (Qureshi *et al.*, 2007).

Ethnobotanical Uses: Annual herb that is non-woody having edible fruits of pleasant strawberry like flavor. It is used to treat profuse mensturation and eye blemishes. Its fruits are antiseptic and used to treat stomach ulcer by mixing with leaves of *Berberis lyceum*. Leaves are diuretic and used in children, s diarrhea. Leaves and roots are used for preparation of tea. Fruits are also laxative and purgative (Qureshi *et al.*, 2007; Ch *et al.*, 2013; Amjad *et al.*, 2015). Ethnobotanical studies from Buhtan reveals that its flowers, fruits and leaves lower the fever (Dorji, 2017).

Previous Studies: Previously this plant has been studies about its antioxidant and antilipidimic activities through invivo and invitro models that shows that it is strong anti-hyperlipidimic and antioxidant (Anees *et al.*, 2018). In a review study there is report about its cytoprotective effects against ischemia-reperfusion induced brain injury in animal model and this protective effect is due to presence of phenolic compounds that have antioxidant properties (Purushottam *et al.*, 2014).

Figure 2.9: *Fragaria nubicola* with Fruits

2.10. 9. *Thymus serphyllum*

Family: Lamiaceae **Varnacular name:** Ban Jawain

Status: Wild herb **Flowering period:** May-August

Ethno-medicinal Uses: It is main medicinal plant of Bagh Azad Kashmir region. Its leaves are laxatives and stomachic. It is used for eye diseases, blood purification, bronchitis and kidney problems. Plant is used for complaints of liver and stomach while its oil is used against toothache (Qureshi *et al.*, 2007). Plant is aromatic, antiseptic, analgesic and diuretic. It is also carminative and stimulant (Aziz and Rehman, 2008).

Previous studies: The extract is reported as antioxidant, antimicrobial and antiseptic (Abramovic *et al.*, 2018). Compound 3-O-β-D glucopyronosyl-sistosterol is effective against HIV (Aziz and Rehman, 2008). Oil obtained from *T. serphyllum* has antioxidant value (Aslam *et al.*, 2012). Effect of *T. serphyllum* extract on apoptosis, cell proliferation and epigenetic events in breast cancer cell are reported (Bozkurt *et al.*, 2012). There is no report about its hepatoprotective effects studies.

Figure 2.10: ***Thymus serphyllum*** **with Flowers**

2.10.10. *Primula macrophylla*

Family: Primulaceae **Vernacular name:** Khakhri

Status: Wild herb **Flowering period:** June-July

Ethno-medicinal Uses: Its flowers and leaves are used for medicinal purposes. It is used as an anti-inflammatory and febrifuge. It is used for treatment of diarrhea, inflammation of liver and stomach problems. In children, gall bladder disorders and fever are treated with it (Shaheen, 2010). Many species of Primula are used as traditional medicines for epilepsy, convulsions, spleen problem, kidney and liver damages (Fouwad *et al.*, 2014).

Previous Studies: There are some reports about its pharmacological investigations. Its antimicrobial and antifungal activities are studied on crude extracts in benzene and ethyl acetate fractions (Najmus-saqib *et al.*, 2009). Anti-leishmanial activity is due to flavonoid compound, 2-phenylchromone (Khaleefa *et al.*, 2014).

Figure 2.11: *Primula macrophylla* with Flower

There are no literature reports regarding these plants under study from Northern localities of Azad Jammu and Kashmir, Himalaya, Pakistan about their hepatoprotective studies through invivo model against CCl_4 induced toxicity.

2.11. Identification Techniques for Phytochemicals

One of the important steps for natural products study is their profiling. Identification of herbal extract is performed by techniques such as chromatography (Shinde *et al.*, 2009). Purification of compounds, separation or extracting the target compound from contaminant is efficiently carried out by High Performance Liquid Chromatography (HPLC). HPLC technique is mainly applied to isolate natural products (Fan *et al.*, 2006). Phytochemical analysis and analytical chemistry utilize this HPLC to separate mixture of compounds (Piana *et al.*, 2013). Health care effects of phenolic acids are concentrated and much literature is available on the analytical methods of phenolic compounds from different foods and other natural sources. Reversed-phase High Performance Liquid Chromatography (RP-HPLC) technique is mostly of analytical approach based. Rebecca and Robbins, (2003) separated and determined sixteen (16) different phenolic compounds utilizing HPLC with diode array detection (DAD) in wine. UPLC- PDA-Q/TOF-MS is applied effectively for determination and the evaluation of *Lepidium sativum* extracts if the substances only occur in low quantity while the combination of retention time and the calculation of accurate molecular mass helps in fast qualitative analysis of compounds (Oszmianski*et al.*, 2013). Handling the compounds of a diverse polarity and molecular mass, its versatility, simplicity and scope of the reversed-phase method; are main reasons of this technique to apply (Boligon and Athayde, 2014). The identification of phytoconstituents gives key idea to know the actual composition of phytochemicals in plant extract that may help for assessment of pharmacological and biological activities of those plants.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. Chemicals and Solvents

All solvents (Methanol, n-Hexane, Chloroform, *n*-Butanol, Ethyl acetate and Ethanol) were of analytical grade while some solvents were HPLC grade (methanol, acetonitrile and orthophosphoric acid 85%) and LCMS grade (Acetonitrile), were purchased from registered chemical companies (Sigma Aldrich).Analytical kits for analysis of serum biochemical parameters i.e. alanine aminotransferase (ALT) also called serum glutamate pyruvate transaminase (SGPT), aspartate aminotransferase (AST) also called serum glutamate oxaloacetic transaminase (SGOT), alkaline phosphatase (ALP) and total bilirubin (TB)) as well as standard drug (Silymarin), were purchased from Merck company. $FeCl_3$, magnesium ribbon, Glacial acetic acid, Folin-Ciocaulate reagent (phosphomolybdate and phosphotungstate), Na_2CO_3, $AlCl_3$, 2, 2 diphenyl-1-picryl-hydrazyl free radical (DPPH), reference standards (for phenolic and flavonoids), hematoxylin and eosin (H&E) purchased were of Sigma-Aldrich company. Other used materials; buffered formalin (10%); xylene, paraffin, bees wax and olive oil were locally purchased.

3.2. Instruments (Equipments and Glassware)

Equipments used were U-V-visible spectrophotometer, thermometer, mechanical grinder, laboratory centrifuge, rotatory evaporator (IKA RV10 control), syringe nylon filter (0.45μm), filter papers (Whatsman No.1), light microscope, microtome, volumetric flasks, beakers, columns, RP-HPLC apparatus, Perkin Elmer Series 200 pump equipped with 200 UV/VIS detectors Symmetry C18, microplate reader spectrophotometer, LC-ESI-Q-TOF-MS apparatus (Agilent technologies Santa Clara, USA), HPLC chromatograph (1260 Series), PDA detector (G1315D) and mass spectrometer (G6530B) for LCMS. Hamilton micro liter syringe, sonicator, oven, digital balance, falcon tubes, Eppendorf tubes, glass vials, cotton and test tubes etc. were used during experiments.

3.3. Study Design

Whole study was designed to study the selected plants for hepatoprotective effects; comprising two parts i.e. Part-I: Screening of the selected medicinal plants for their hepatoprotective effect and Part-II: Investigation of most active hepatoprotective plants.

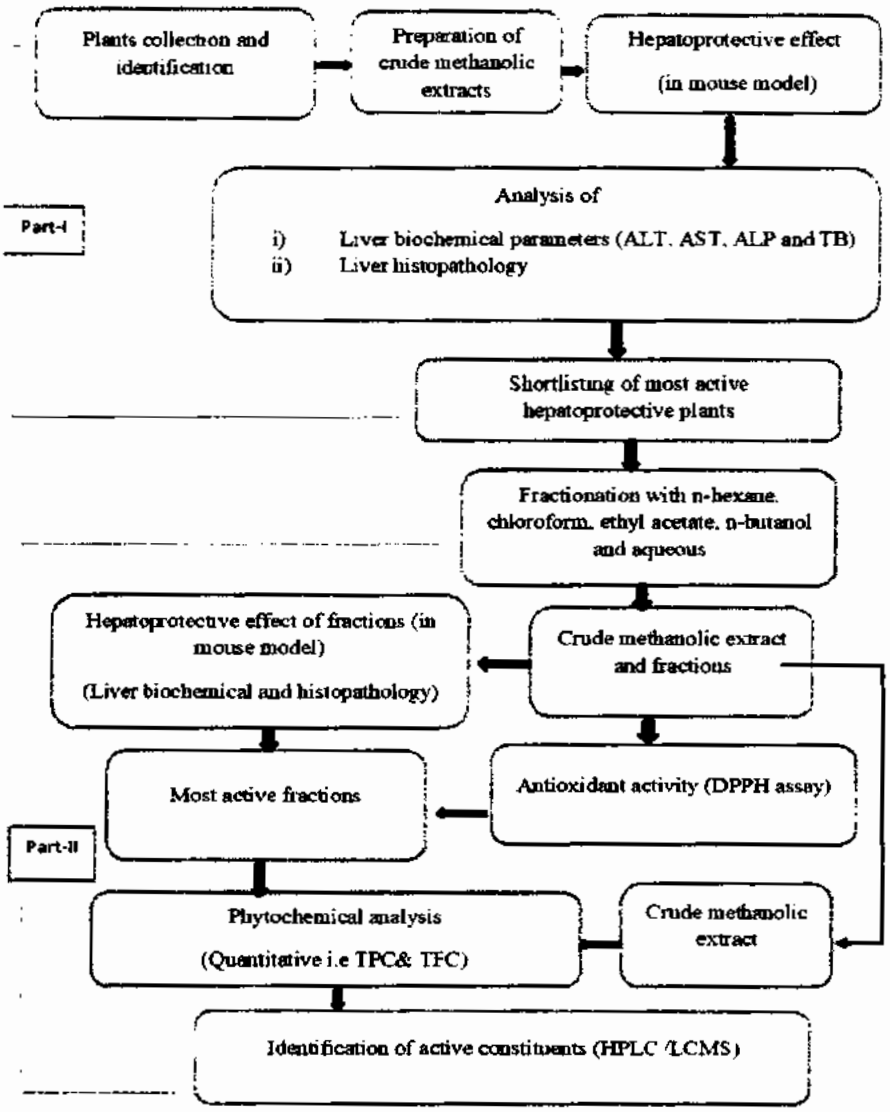


Figure 3.1: Experimental Design

3.4. Plant Material

3.4.1. Selection and Collection of Plants Material

Initially ten (10), ethno medicinally important plants (P.1- P.10), based on their medicinal importance and their uses by folk people of that area were selected. Selected plants were collected from various localities of Poonch Division (Bagh, Haveli and Poonch) of Azad Jammu and Kashmir Pakistan, according to their seasonal availability (April to September). Herbarium sheets of plants were prepared by pressing, drying and mounting after collection.

3.4.2. Identification of Plant Samples

Dr. Aneel Gilani, Associate Curator, Botanical Science Division Pakistan museum of natural history (PMNH) Islamabad, Pakistan was consulted for identification of the plants. Correctly identified specimens; *Viola canescens*, *Dryopteris ramosa*, *Geranium wallichianum*, *Eleagnus parvifolia*, *Rosa brunonii*, *Aconogonon alpinum*, *Oxyria digyna*, *Fragaria nubicola*, *Thymus serphyllum* and *Primula macrophylla* were deposited as voucher specimens (040948, 040931, 040996, 040998, 040988, 040991, 040997, 040943, 040993 and 040995) respectively for future references in the herbarium of PMNH Islamabad.

Table 3.1: List of the Plants and Their Parts Used Under Study

S.NO	Botanical Name	Local Name	Family	Parts used
P. 1	*Viola canescens*	Banafsha	Violaceae	Flowers
P. 2	*Dryopteris ramosa*	Kunji	Dryopteridaceae	Leaves
P. 3	*Geranium wallichianum*	Rattenjot	Gerinaceae	Roots
P. 4	*Elaeagnus parvifolia*	Kankoli	Elaeagnaceae	Fruits
P. 5	*Rosa brunonnii*	Tarni	Rosaceae	Flowers
P. 6	*Aconogonon alpinium*	Masloon	Polygonaceae	Roots
P. 7	*Oxyria digyna*	Khutry	Polygonaceae	Shoots
P. 8	*Fragaria nubicola*	Budmeva	Rosaceae	Whole plant
P. 9	*Thymus serphyllum*	Banjwain	Lamiaceae	Shoots
P. 10	*Primula macrophylla*	Khakhri	Primulaceae	Whole plant

3.5. Preparation of Methanolic Extract (ME)

3.5.1. Grinding of Plant Material

After washing the plants material (parts used for study) with clean water, were air dried at room temperature under the shade-conditions. Samples were grinded into powder form using mechanical grinder. Each plant's sample was weighted separately and packed in zipped polythene bags to avoid moisture and contamination.

3.5.2. Extraction Procedure

Initially, ME of ten-selected medicinal plant samples were prepared according to well established protocols (Shah *et al.*, 2012; Khan *et al.*, 2012; Barkatullah *et al.*, 2011). Briefly, dried powdered materials (120 g each) of each plant sample was added to methanol in ratio of 1:3 in ten separate round bottom flasks of 1L (1000 mL) each and were kept for soaking for 6 days at room temperature. Suspension of each plant was stirred daily for smooth homogenization to dissolve completely. Then the samples of each plant were filtered through filter papers separately. In the residue of each plant sample; 300 mL analytical grade methanol was separately added; kept for four additional days and then filtered each sample on 5th day. Extract of each plant was concentrated using rotatory evaporator at 40 °C under reduced pressure. Hence, these extract were methanolic extract (ME) of each plant (P.1-P.10) respectively of the selected plants. Extract of each plant was weighted after drying. The extracts of the plants were labelled as crude methanolic extract of *Viola canescens* flowers (VCME), crude methanolic extract of *Dryopteris ramosa* leaves (DRME), crude methanolic extract of *Geranium wallichianum* rhizome (GWME), crude methanolic extract of *Elaeagnus parvifolia* fruits (EPME), crude methanolic extract of *Rosa brunonii* flowers (RBME), crude methanolic extract of*Aconogonon alpinium* roots (AAME), crude methanolic extract of *Oxyria digyna* shoots (ODME), crude methanolic extract of *Fragaria nubicola* whole plant (FNME), crude methanolic extract of *Thymus serphyllum* shoots (TSME) and crude methanolic extract of *Primula macrophylla* whole plant (PMME) for plant one to plant ten (P.1-P.10) respectively.

3.6. Hepatoprotective Activity of Methanolic Extract (ME) of Selected Plants

Hepatoprotective effect of crude methanolic extracts of preliminary selected plants was performed according to standard procedure given below:

3.6.1. Experimental Animals

BALB/C albino mice (both sex), of uniform weight (30±5) were purchased from animal house of National Institute of Health (NIH) Islamabad. Mice were maintained at 25±5

°C for 12 hrs light/dark cycles, in stainless steel cages in animal's experimental room of NIH Islamabad. Animals were allowed to acclimatize before start of experimental procedure for 7 days with provision of normal diet and water access.

3.6.2. Oral Toxicity Test

Acute oral toxicity study of all plants was performed according to guidelines of "Organization for Economic Co-operation and Development (OECD-425)" with slight modifications (Kiran *et al.*, 2012). Limit test dose of 2000mg/kg was orally administered in animals. For this purpose, 55 female BALB/C albino mice of uniform weight (30±5 g) were selected and then were divided into 11 groups (G-I to G-XI).

Group-I; was normal control while group G-II- G-XI were treated with methanolic extract dose (2000 mg/kg) of selected plants (P1-P10) respectively. One mouse of each group (group G-II to G-XI) was fasted overnight having access to drinking water. These ten animals (one fasted in each group) were given 2000 mg/kg of test extract of each plant (plant-1 extract in group-II to plant-10 extract in group-XI) respectively. Animals were observed for 24hr for mortality. Animals in all treated groups (G-II-GXI) survived and then four additional animals in each treated group (Group-II-XI) were tested sequentially with test extracts of plants (P.1-P.10) respectively so that 5 animals were tested of each group (total of 50 animals in all 10 groups). All animals were closely observed initially 30 minutes after dosing and then periodically for early 24 hours. The behavior changes such as hyperactivity, atoxia, tremors, convulsions, salvations, diarrhea, coma, lethargy and sleep were observesved. All animals were observed daily for 14 days (Kiran *et al.*, 2012).

3.6.3. Hepatoprotective Effect of ME of Selected Medicinal Plants Against CCl_4 Induced Hepatotoxicity

Dose Selection: One-tenth (1/10th) and one-fifth ($1/5^{th}$) of maximum tolerated dose (2000 mg/kg) of each plant extract tested for acute oral toxicity, was selected for further experimental process for hepatoprotective activities (Hussain *et al.*, 2012).

Dose Calculations: Each plant methanolic extract was dissolved separately in normal saline (0.9 %) solution. Dose calculation was made according to guideline laid by OECD-423, which is described as following.

Normal Saline Solution Calculation: Daily saline dose required for each mouse of 30 g was 0.6 mL. For six mice in each group was 3.6 mL saline solution required. Total dose administered for 28 days was 100.8 mL. One (1) mouse of 30 g for each day required 0.6 mL and for six mice in each group for each day required 6x 0.6= 3.6 mL. For 28 days, six mice required 3.6 x28 = 100.8 mL.

Crude Extract Dose Calculation: For 30 g mouse, required dose of extract was 12 mg (400 mg/kg) for each day. For six mice in each group, required dose was 72 mg and for 28 days, the required dose of each plant extract was 2016 mg. Total 2016 mg (2.16 g) of each plant ME was independently weighed and transferred to 150 mL each, 10 falcon tubes separately. Then 100.8 mL saline solution was transferred in each falcon tube by syringe. Sonicator was used for efficient dissolving of each extract. As the treatment were of two levels of dose i.e. 200mg/kg and 400 mg/kg for each plant extract in separate groups. For low dose (200mg/kg) treatment for 6 mice, 50% dilution was made with 0.9 % saline solution for each plant extract solution of 12 mg (400mg/kg).

Standard Drug Dose Calculation: Dose of standard hepatoprotective drug (Silymarin) for 6 animals of standard group (GIII) with 100 mg/kg b.w was calculated (Afzal *et al.*, 2013). One mouse-required dose was 3 mg/d, for 6 mice, required dose was 18 mg for one day and for 28 days was 504mg. Total of 504mg of standard drug weighed independently and then transferred to 150 mL falcon tube. Now 100.8 mL of normal saline solution was added in the falcon tube by syringe and then dissolved the drug by sonicator efficiently.

3.6.4. Experimental Design for Hepatoprotective Activities

Experiments on mice was performed under the approval of "Institutional Bioethics and Biosafety Committee (IBBC)" of International Islamic University (IIU) Islamabad; No. IIU BI&BT/FBAS-IBBC-2015-04, dated June 30, 2015, and according to guideline laid by OECD-423 (adopted on 17th December 2001). Total 138 animals (male mice) were divided

into twenty-three (23) groups (n= 6 in each group). Treated Groups (G-IV to G-XXIII) were given treatments of 200 mg/kg and 400 mg/kg methanolic extract of each plant (plant-1 to plant-10) respectively. All groups were treated for 28 days as mentioned below. SD (Silymarin 100 mg/kg) to G-III was given according to protocols followed by Afzal *et al.*, (2013) and Saleem *et al.*, (2014) with little modifications. Carbon tetrachloride (CCl_4 25% in olive oil) having 1mL/kg was induced according to Singhal and Gupta, (2012) and Afzal *et al.*, (2013). Water and normal feed was provided to all animals daily in all groups. The treatment was performed by the following procedure described below.

3.6.5. Treatment Procedure

G-I: (Normal control) received only water with normal feed for 28 days. G-II: (Negative /Toxicant control group): Carbon tetrachloride (25% diluted in olive oil) was injected (1mL/kg) through intraperitoneal injections with interval of two days for 13 days. G-III: (SD group): Carbon tetrachloride (CCl_4) like G-II+SD (Silymarin 100mg/kg/ d for 28 days. G-IV: Carbon tetrachloride (CCl_4) like G-II+methanolic extract of plant one (VCME); with oral administration of 200mg/kg/d up to 4 weeks. G-V: Carbon tetrachloride (CCl_4 25%) like G-II+methanolic extract of plant one (VCME); with oral administration of 400mg/kg)/d up 4 weeks. G-VI: Carbon tetrachloride (CCl_4 25%) like G-II+methanolic extract of plant two (DRME); with oral administration of 200mg/kg/d up to 4 weeks. G-VII: Carbon tetrachloride (CCl_4 25%) like G-II+methanolic extract of plant two (DRME); with oral administration of 400mg/kg/d up to 4 weeks. G-VIII: Carbon tetrachloride (CCl_4 25%) like G-II+methanolic extract of plant three (GWME); with oral administration of 200 mg/kg/d up to 4 weeks. G-IX: Carbon tetrachloride (CCl_4 25%) like G-II+methanolic extract of plant three (GWME); with oral administration of 400 mg/kg/d up to 4 weeks. G-X: Carbon tetrachloride (CCl_4 25%) like G-II+methanolic extracts of plant four (EPME); with oral administration of 200 mg/kg/d up to 4 weeks. G-XI: Carbon tetrachloride (CCl_4 25%) like G-II+methanolic extract of plant four (EPME); with oral administration of 400 mg/kg/d up to 4 weeks. G-XII: Carbon tetrachloride (CCl_4 25%) like G-II+methanolic extract of plant five (RBME); with oral administration of 200 mg/kg/d up to 4 weeks. G-XIII: Carbon tetrachloride (CCl_4 25%) like G-II+methanolic extract of plant five (RBME); with oral administration of 400 mg/kg/d up to 4 weeks. G-XIV: Carbon tetrachloride (CCl_4 25%) like

G-II+methanolic extract of plant six (AAME); with oral administration of 200 mg/kg/d up to 4 weeks. G-XV: Carbon tetrachloride (CCl_4 25%) as in group-II+treatment with methanolic extract of plant six (AAME); oral administration of 400 mg/kg/d up to 4 weeks. G-XVI: Carbon tetrachloride (CCl_4 25%) like G-II + methanolic extract of plant seven (ODME); with oral administration of 200 mg/kg/d up to 4 weeks. G-XVII: Carbon tetrachloride (CCl_4 25%) like G-II + methanolic extract of plant seven (ODME); with oral administration of 400 mg/kg/d up to 4 weeks. G-XVIII: Carbon tetrachloride (CCl_4 25%) like G-II+methanolic extract of plant eight (FNME); with oral administration of 200 mg/kg/d up to 4 weeks. G-XIX: Carbon tetrachloride (CCl_4 25%) like G-II+methanolic extract of plant eight (FNME); oral administration of 400 mg/kg up to 4 weeks. G-XX: Carbon tetrachloride (CCl_4 25%) like G-II+methanolic extract of plant nine (TSME); oral administration of 200 mg/kg/d up to 4 weeks. G-XXI: Carbon tetrachloride (CCl_4 25%) like G-II+methanolic extract of plant nine (TSME); with oral administration of 400 mg/kg/d up to 4 weeks. G-XXII: Carbon tetrachloride (CCl_4 25%) like G-II+methanolic extract of plant ten (PMME); with oral administration of 200 mg/kg/d up to 4 weeks. G-XXIII: Carbon tetrachloride (CCl_4 25%) like G-II+methanolic extract of plant ten (PMME); with oral administration of 400 mg/kg/d up to 4 weeks.

Figure 3.2: Mice Sorted for Analysis of Hepatoprotective Effect of Selected Plants Extracts

3.7. Biochemical and Histopathological Investigations of Mice

Food was stopped 12 hours before sacrificing of mice (Kumaresan *et al.*, 2015). On 29th day, mice were sacrificed with anesthetizing them to analyze biochemical markers and histological studies of livers for each group.

Blood Collection: Blood was collected in plan glass tubes by syringes via cardiac puncture and then allowed to clot for 40-45 min. Then blood serum separated by centrifugation (3000 rpm for 15 min), was preserved at 4 °C for further assay.

Tissue Sampling: Livers slices were also separated by disection, then washed with water; added (fixed) in buffered formalin (10%) for 24 hours untilthe histopathological analysis.

Figure 3.3: Blood Collection

Figure 3.4: Mice Livers Preserved in Formalin (10%)

3.7.1. Biochemical Assay

Liver marker enzymes i.e alanine aminotransferase(ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) activities were estimated spectrophotometrically using diagnostic kits (Merck and Diasys Diagnostic system Gmbh Germany) with standard method (Hussain *et al.*, 2014). Similarly, serum total bilirubin (TB mg/dL) was estimated (Parma *et al.*, 2010). Percentage (%) protection of liver biochemical parameters was calculated by following equation.

Percentage (%) protection = mean value of CCl_4 control - Mean value of test sample ÷ mean value of CCl_4 control − Mean value of normal control, as described by Rawat *et al.*, (1997).

3.7.2. Histopathological Studies

Dehydration of liver specimens was performed by different dilutions of ethyl alcohol (70%, 80% and 100%) and then cleared in xylene. Livers sectioning of 4-5 µm thickness were prepared through sledge microtome by embedding the tissue in paraffin bees wax blocks, fixed in hot air oven at 56 °C for 24hours (Ashoush *et al.*, 2013). Sections were mounted on slides and after tissue fixation, staining with hematoxylin-eosin (H&E) was done for histological examinations under microscope to see the changes in liver hepatocytes (Ashoush *et al.*, 2013; Saleem *et al.*, 2014). Photographs of slides were taken by camera.

3.7.3. Short Listing of Most Active Hepatoprotective Plants

Based on results for hepatoprotective activity of 10 selected plants against CCl_4 induced toxicity in mice; plants thatexhibited highest significant effects were selected for further analysis.

3.8. Investigation of Short Listed Most Active Hepatoprotective Plants

3.8.1. Extraction of Short Listed MostActive Plants

Plant material (powder form) of short listed active plants i.e. *G. wallichianum*, *E. porvofolia* and *A. alpinum* (A, B & C respectively) was again processed for preparation of more quantity of crude methanolic extract. 2 kg (2000 g) of each plant material (powder form) was separately treated extraction process. For this purpose, 2x1kg (2x1000g) of each

plant material was processed for soaking in methanol (3000 mL) in 5 liters' flasks (round bottom) separately; allowed to stand for 6 days with occasionl shaking. Then sample of each plant was separately filtered using filter papers (What Man filter paper). Merc of each selected plant sample was again added in 3000 mL methanol separately and kept for additional 4 days, which were again filtered separately. Now concentrated the whole extract of each plant using rotatory evaporator at 40 °C under reduced pressure. The plants extracts were assigned number i.e. *G. walichianum* methanolic extract (GWME*)* asplant A (P-A), *E. porvofolia* methanolic extract (EPME) as plant B (P-B) and *A. alpinum* methanolic extract (AAME) as plant C (P-C). Extract of each plant was weighted after drying.

3.9. Fractionation of Short Listed Most Active Plants Extracts (GWME, EPME and AAME) and Their Hepatoprotective Activities

3.9.1. Fractionation Process of GWME, EPME and AAME

The methanolic extract of three short listed plants (*Geranium walichianum*, *Eleagnus parvofolia* and *Acconogonon alpinum*) was fractionated with different organic solvents on increasing polarity basis (Ihsan-ul-Haq *et al.*, 2012). The fractionation of each plant extract was made separately using different solvents (n-hexane, chloroform, ethyl acetate, n-butanol and water) based on increasing polarity. Methanolic extract (200g) of each plant (*G. walichianum*, *E. parvofolia* and *A. alpinum*) was suspended in distilled water (200mL) separately, which formed the water suspension of methanolic extract of each plant.

The aqueous suspensions of each plant extract was partitioned to five fractions i.e. n-hexane fraction (HF), chloroform fraction (CF), ethyl acetate fraction (EF), n-butanol fraction (BF) and an aqueous fraction (AF). For this purpose; added n-hexane (3x200mL) into water suspensions and shaked well, which formed two layers (upper n-hexane layer and lower an aqueous layer). Then n-hexane layer was separated by separating funnel of each plant extract. It was dried by rotatory evaporator at 40 °Cunder reduced pressure. This was n-hexane fraction (HF). In the aqueous portions, chloroform (3x200 mL) was added, shaken well in separating funnel, which developed an upper layer of water and lower layer of chloroform. This layer separated as chloroform fraction (CF). It was evaporated and dried by rotatory evaporator at 40 °C under reduced pressure. Now residue remained as an aqueous

portion. Then ethyl acetate (3x200 mL) was added. Two layers were separated by separating funnel for each extract and the separated upper layers were of ethyl acetate that was dried by rotatory evaporator at 40 °C under reduced pressure. This was ethyl acetate fraction (EF). Later on n-butanol (3x200 mL) was added in the remaining aqueous portions of each extract. Again, there were developed two layers in each separating funnel, an upper portion of n-butanol and lower layer of an aqueous portion of extract. These were separated into n-butanol fractions (BF) and aqueous fractions (AF). Then dried each fraction by rotatory evaporator at 40 °Cunder-reduced pressure.

All the fractions of three selected plants were weighed separately and labeled properly. These were labelled as: For *G. wallichianum*; crude methanolic extract of *G. wallichianum* (GWME), *G. wallichianum* n-hexane fraction(GWHF), *G. wallichianum* chloroform fraction (GWCF), *G. wallichianum* ethyl acetate fraction (GWEF), *G. wallichianum* n-butanol fraction (GWBF) and *G. wallichianum* aqueous fraction (GWAF). Similarly, for *E. parvifolia*; crude methanolic extract of *E. parvifolia* (EPME), *E. parvifolia* n-hexane fraction (EPHF), *E. parvifolia* chloroform fraction (EPCF), *E. parvifolia* ethyl acetate fraction (EPEF), *E. parvifolia* n-butanol fraction (EPBF) and *E. parvifolia* aqueous fraction (EPAF). For *A. alpinum*: crude methanolic extract of *A. alpinum* (AAME), *A. alpinum* n-hexane fraction (AAHF), *A. alpinum* chloroform fraction (AACF), *A. alpinum* ethyl acetate fraction (AAEF), *A. alpinum* n-butanol fraction (AABF) and *A. alpinum* aqueous fraction (AAAF). All the extracts were stored at 4 °C to prevent from contaminations until further analysis.

Figure 3.5: Fractionation of Methanolic Extract (ME) of Active Plants

Crude Methanolic Extract (ME) = 200 g

↓ Suspension in Distilled Water (200 mL)

Water Suspension of ME

↓ Extraction with n-Hexane (3x 200 mL) ⟶ n-Hexane Fraction (HF)

Aqueous Portion

↓ Extraction with Chloroform (3x 200 mL) ⟶ Chloroform Fraction (CF)

Aqueous Portion

↓ Extraction with Ethyl Acetate (3x 200 mL) ⟶ Ethyl Acetate Fraction (EF)

Aqueous Portion

↓ Extraction with n-Butanol (3x 200 mL) ⟶ n-Butanol Fraction (BF)

Aqueous Fraction (AF)

Figure 3.6: General Schematic for Fractionation ofME of Selected Active Plants

3.10. Hepatoprotective Effect of Fractions of Short Listed Active Plants (*G. walichianum*, *E. parvofolia* and *A. alpinum*)

3.10.1. Experimental Animals

During this analysis; 198 male albino mice (BALB/C) of average weight (30±5g), purchased from animal house of National Institute of Health (NIH) Islamabad; were housed in cages providing same environmental conditions as described in 3.6.1.

3.10.2. Dose Selection and Calculation

The dose of 200 mg/kg & 400 mg/kg of each fraction of each plant extract was selected to study the effects of different fractions of selected active plants. Dose of each fraction of selected plant was calculated in same way as in previous experimental work.

3.10.3. Experimental Design

Five fractions i.e n-hexane (HF), chloroform (CF), ethyl acetate (EF), n-butanol (BF) and aqueous fractions (AF) of methanolic extracts of each short listed active plants were tested for their hepatoprotective effects. Mice were grouped according to previous procedure of Ahsan *et al.*, (2009) and More *et al.*, (2013) with some modifications. Mice under study were comprised thirteen groups (n=6 in each) for each plant fractions activities. For each plant having normal control group (G-I), CCl_4 toxicant /negative control (G-II) and positive/standard drug control(G-III) were common while the mice in groups IV to XIII were comprised into IVA to XIIIA, IVB to XIIIB and IVC to XIIIC for treatment with *G. wallichianum* fractions, *E. parvifolia* fractions and *A. alpinum* fractions respectively. Normal feed and water was provided to all animals for four weeks (28 days).

3.10.4. Treatment Procedure with Different Fractions of Most ActiveHepatoprotective Plants

Group-I: (NC); was provided free access for normal food and water up to 28 days. Group-II: Toxicant control group: 25% Carbon tetrachloride CCl_4 (with dilution in olive oil, 1 mL/kg body weight) was administrated through intraperitoneal injections with two days' interval up to 13 days. G-III: (SD group): Carbon tetrachloride (CCl_4) like G-II+ SD (100

mg/kg) for four (4) week. Animals in Groups IVA- XIIIA, IVB- XIIIB and IVC- XIIIC were treated as described below.

A: Treatment with *G. wallichianum* Fractions: Group-IVA: Carbon tetrachloride (CCl_4 25%) like G-II+n-hexane fraction of *G. wallichianum* (GWHF); oral administration of 200 mg/kg/d, up to four weeks (twenty-eight days). Group-VA: Carbon tetrachloride (CCl_4 25%) like G-II+n-hexane fraction of *G. wallichianum* (GWHF); oral administration of 400 mg/kg/d, up to four weeks (twenty-eight days). Group-VIA: Carbon tetrachloride (CCl_4 25%) like G-II+chloroform fraction of *G. wallichianum* (GWCF); oral administration of 200 mg/kg/d, up to four weeks (twenty-eight days). Group-VIIA: Carbon tetrachloride (CCl_4 25%) like G-II+chloroform fraction of *G. wallichianum* (GWCF); oral administration of 400 mg/kg/d up to four weeks (twenty-eight days). Group-VIIIA: Carbon tetrachloride (CCl_4 25%) like G-II+ethyl acetate fraction of *G. wallichianum* (GWEF); oral administration of 200 mg/kg/d, up to four weeks (twenty-eight days). Group-IXA: Carbon tetrachloride (CCl_4 25%) like G-II+ethyl acetate fraction of *G. wallichianum* (GWEF); oral administration of 400 mg/kg/d, up to four weeks (twenty-eight days). Group-XA: Carbon tetrachloride (CCl_4 25%) like G-II+n-butanol fraction of *G. wallichianum* (GWBF); oral administration of 200 mg/kg/d, up to four weeks (twenty-eight days). Group-XIA: Carbon tetrachloride (CCl_4 25%) like G-II+n-butanol fraction of *G. wallichianum* (GWBF); oral administration of 400 mg/kg/d, up to four weeks (twenty-eight days). Group-XIIA: Carbon tetrachloride (CCl_4 25%) like G-II+aqueous fraction of *G. wallichianum* (GWAF); oral administration of 200 mg/kg/d, up to four weeks (twenty-eight days). Group-XIIIA: Carbon tetrachloride (CCl_4 25%) like G-II+aqueous fraction of *G. wallichianum* (GWAF); oral administration of 400 mg/kg/d, up to four weeks (twenty-eight days).

B: Treatment with *E. parvifolia* Fractions: Group-IVB: Carbon tetrachloride (CCl_4 25%) like G-II+n-hexane fraction of *E. parvifolia* (EPHF); oral administration of 200 mg/kg/d, up to four weeks (twenty-eight days). Group-VB: Carbon tetrachloride (CCl_4 25%)like G-II+n-hexane fraction of *E. parvifolia* (EPHF); oral administration of 400 mg/kg/d, up to four weeks (twenty-eight days). Group-VIB: Carbon tetrachloride (CCl_4 25%) like G-II+chloroform fraction of *E. parvifolia* (EPCF); oral administration of 200 mg/kg/d, up to four weeks

(twenty-eight days). Group-VIIB: Carbon tetrachloride (CCl_4 25%)like G-II+chloroform fraction of *E. parvifolia* (EPCF); oral administration of 400 mg/kg/d, up to four weeks (twenty-eight days). Group-VIIIB: Carbon tetrachloride (CCl_4 25%) like G-II+ethyl acetate fraction of *E. parvifolia* (EPEF); oral administration of 200 mg/kg/d, up to four weeks (twenty-eight days). Group-IXB: Carbon tetrachloride (CCl_4 25%) like G-II+ethyl acetate fraction of *E. parvifolia* (EPEF); oral administration of 400 mg/kg/d once daily, up to four weeks (twenty-eight days). Group-XB: Carbon tetrachloride (CCl_4 25%) like G-II+n-butanol fraction of *E. parvifolia* (EPBF); oral administration of 200 mg/kg/d, up to four weeks (twenty-eight days). Group-XIB: Carbon tetrachloride (CCl_4 25%) like G-II+n-butanol fraction of *E. parvifolia* (EPBF); oral administration of 400 mg/kg/d, up to four weeks (twenty-eight days). Group-XIIB: Carbon tetrachloride (CCl_4 25%) like G-II+aqueous fraction of *E. parvifolia* (EPAF); oral administration of 200 mg/kg/d, up to four weeks (twenty-eight days). Group-XIIIB: Carbon tetrachloride (CCl_4 25%) like G-II+aqueous fraction of *E. parvifolia* (EPAF); oral administration of 400 mg/kg/d, up to four weeks (twenty-eight days).

C: Treatment with *A. alpinum* Fractions: Group-IVC: Carbon tetrachloride (CCl_4 25%)like G-II+n-hexane fraction of *A. alpinum* (AAHF); oral administration of 200 mg/kg/d, up to four weeks (twenty-eight days). Group-VC: Carbon tetrachloride (CCl_4 25%) like G-II+n-hexane fraction of *A. alpinum* (AAHF); oral administration of 400 mg/kg/d, up to four weeks (twenty-eight days). Group-VIC: Carbon tetrachloride (CCl_4 25%) like G-II+chloroform fraction of *A. alpinum* (AACF); oral administration of 200 mg/kg/d, up to four weeks (twenty-eight days). Group-VIIC: Carbon tetrachloride (CCl_4 25%) like G-II+chloroform fraction of *A. alpinum* (AACF); oral administration of 400 mg/kg/d, up to four weeks (twenty-eight days). Group-VIIIC: Carbon tetrachloride (CCl_4 25%) like G-II+ethyl acetate fraction of *A. alpinum* (AAEF); oral administration of 200 mg/kg/d, up to four weeks (twenty-eight days). Group-IXC: Carbon tetrachloride (CCl_4 25%) like G-II+ethyl acetate fraction of *A. alpinum* (AAEF); oral administration of 400 mg/kg/d, up to four weeks (twenty-eight days). Group-XC: Carbon tetrachloride (CCl_4 25%) like G-II+n-butanol fraction of *A. alpinum* (AABF); oral administration of 200 mg/kg/d, up to four weeks (twenty-eight days). Group-XIC: Carbon tetrachloride (CCl_4 25%) like G-II+n-butanol

fraction of *A. alpinum* (AABF); oral administration of 400 mg/kg/d, up to four weeks (twenty-eight days). Group-XIIC: Carbon tetrachloride (CCl_4 25%)l ike G-II+aqueous fraction of *A. alpinum* (AAAF); oral administration of 200 mg/kg/d, up to four weeks (twenty-eight days). Group-XIIIC: Carbon tetrachloride (CCl_4 25%) like G-II+aqueous fraction of *A. alpinum* (AAAF); oral administration of 400 mg/kg/d, up to four weeks (twenty-eight days). After last doses administration of all the fractions in respective groups; mice were stoped to feed and water access 12 hours before their sacrifice. Blood was collected; animals were anesthetized and then sacrificed to isolate the livers.

3.11. Biochemical Parameters and Liver Histopathological Analysis of Mice After Treatment with Fractions of *G. wallichianum*, *E. Parvifolia* and *A. alpinum*

Collected blood was put into glass tubes, allowed to stand for cloting upto 45 min. Blood serum was estimated for biochemical parameters according to standard method (Tahir *et al.*, 2008) after its separation by centrifugation (3000 rpm; 20 min). Livers were removed and processed with same procedure as described in 3.7 and 3.7.2 according to standard procedure (Ashoush *et al.*, 2013).

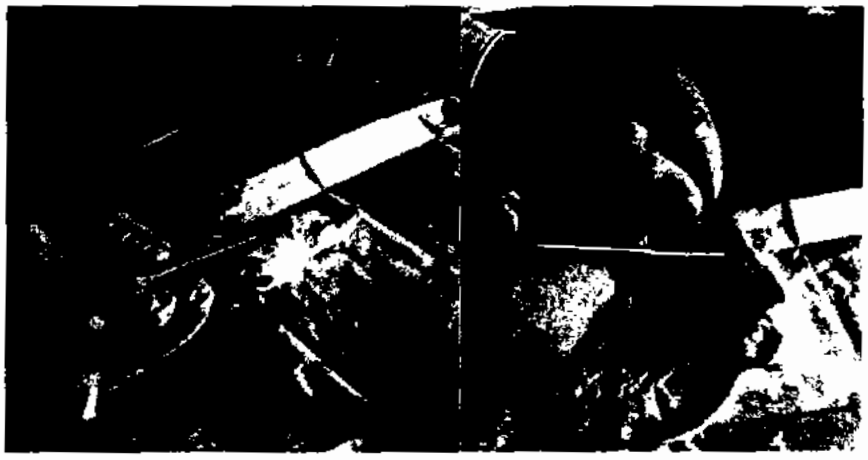


Figure 3.7: Blood Collection and Livers Sampling After Treatment with Different Fractions of Most Active Plants

3.11.1. Biochemical Assay

Liver marker enzymes i.e. alanine aminotransferase (ALT), aspartate amino transferase (AST) and alkaline phosphatase (ALP) activities were estimated spectrophotometrically using diagnostic kits (Merck Germany) with standard procedures (Tahir *et al.*, 2008; Hussain *et al.*, 2014). Serum total bilirubin (TB) level was also estimated according to method described by Parma *et al.*, (2010).

3.11.2. Histological Studies

Livers were processed with same standard procedure as described in 3.7.2. Sections were fixed, mounted on slides and then staining with heamatoxylin-eosin was done for histological examinations under microscope to see the changes in liver hepatocytes (Ashoush *et al.*, 2013; Saleem *et al* 2014; Afzal *et al.*, 2013). Photomicrographs of slides were taken for analysis of liver architecture.

Figure 3.8: Livers Preserved in Formalin (10%) After Treatment with Fractions of Short Listed Active Plants

3.11.3 Determination of Most active fractions

On basis of results of hepatoprotective investigation of different fractions of selected active plants, the active fractions were determined.

3.12. Bioactivity Determination (Antioxidant Activity by DPPH Assay)

Various bioactivities of chemical constituents of plants are described, which include anti-inflammatory, analgesics and antioxidant, in which antioxidant activity is the important one (Cai *et al.*, 2004; Wojdylo *et al.*, 2007). Antioxidant activity of crude extracts as well as the most active fractions of selected active plants was performed with method of Kulisic *et al.*, (2004) with some modifications using DPPH (2, 2 diphenyl-1-picryl-hydrazyl) as free radical.

3.12.1. DPPH Assay and Calculation of IC_{50}

Antioxidant activity of methanolic extract and the fractions of shortlisted active plants was find out by using DPPH with standard procedure (Kulisic *et al.*,2004; Obied *et al.*, 2005). DPPH (4mg) in methanol (100mL) was dissolved to prepare its solution. The solution of DPPH (2800 µL) mixed in extract solution (200 µL) by addition into glass vials; leading to final concentrations (100, 70, 50, 25, 15 and10 (µg/mL) respectively. After well shaking all the mixtures: kept them at room temperature (25 to 28°C) until one hour. Measured the absorbance at 517nm spectrophotometrically. Methanol as blank was used while DPPH solution (2800 µL) and mixture of methanol (200 µL) as negative control while ascorbic acid was used as positive control. Percentage inhibition (%) was counted according to formula given below and value of IC_{50} was calculated by graphical method of linear regression. $Y=0.7283x+26.76$ with $R^2 = 0.9771$.

$$\text{Scavenging effect (\%A A)} = [(A_C - A_S) \div A_C] \times 100$$

"**Ac**" means the absorbance of negative control and "**As**" means the absorbance of test sample. Same methodology was also adopted to determine antioxidant activity of all the fractions of active plants.

3.13. Phytochemical Analysis (Quantitative Phytochemical Analysis)

Quantitative analysis of methanolic extract as well as most active fractions of short listed hepatoprotective active plants (*G. wallichianum*, *E. parvifola* and *A. alpinum*) were subjected for analysis of total phenolic and total flavonoids contents. Analysis was performed by standard methods with some modifications (Gursoy *et al.*, 2009).

3.13.1. Assay for Total Phenolic Content (TPC)

Assay for total phenolic content was estimated by Folin-Ciocalteu method (Gursoy *et al.*, 2009) at microscale with some modifications. The absorbance was measured at 544 nm using standard Gallic acid (GA). Different concentrations (500, 250, 125, 50, 25,10,5 and 2.5 (µg/mL) ofstandard (GA) solution were prepared by serial dilution. Similarly, samples solutions of plants were prepared also. Analysis of plants samples and Gallic acid solutions were performed with same fashion by measuring the absorbance at 544 nm. Gallic acid calibration curve was plotted with the values of difference between absorbance of tested Gallic acid solutions and the blank absorbance of Gallic acid solutions. During this assay, extract solution (4 µL) +distilled water (180 µL) was added in Folin-Ciocalteu reagent (4 µL) afterwards (in triplicate ways using 96-well plate) for each extract sample, shaking plate vigorously by addition of reagent. Three minutes later, added aqueous sodium carbonate (2%) solution (12 µL), allowed the mixture to stand for 2h in dark with intermittent shaking. Blank sample (extract solution 4µL) +distilled water (180 µL) and of aqueous sodium carbonate solution (12 µL), was used against the test samples for measurement of absorbance at 544 nm. Calculations for phenolic compounds concentrations were performed by the equation: $y = 0.0043X+0.0888$; $R^2 = 0.9879$, constructed from standard Gallic acid calibration curve. Expression of results was done as microgram (µg) per milli gram (mg) of gallic acid equivalent (GAE) i.e. µg/mg GAE of dry extract.

3.13.2. Assay for Total Flavonoid Content (TFC)

Total flavonoid content (TFC) was determined using aluminum chloride method (Gursy *et al.*, 2009) with little modifications using the Quercetin (Q) as refrence standard. Quercetin concentrations (500 µg/mL, 250 µg/mL, 125 µg/mL, 50 µg/mL, 25 µg/mL, 10 µg/mL, 5 µg/mL and 2.5 µg/mL) solutions in methanol were made prepared. Standard

solutions and samples were analysed by use of $AlCl_3$ (2%) solution. Absorbance was measured at 405nm against the blank solution of quercetin. Calibration curve was plotted of the values obtained by subtracting the blank values from the values of tested quercetin solutions. During this process, extract solution (100 µL) was added in 100 µL $AlCl_3$ (2%) solution (performed all this in replicates of three, using 96-well plate). Measured the absorbance at 405nm after 10min against blanks samples (100 µL extract solution+100 µL methanol). Flavonoids concentrations in the samples were calculated suing the equation: $y = 0.0051x + 0.0912$; $R^2 = 0.9964$, constructed from calibration curve of standard quercetin. Expressed the results obtained as microgram (µg) per milligram (mg) quercetin equivalent (QE) i.e. µg/mg QE of dry extract.

3.14. Identification of Compounds

3.14.1 HPLC Analysis

HPLC analysis of methanolic extracts and most active fractions of short listed active plants was done by co-elusion of samples with reference standard compounds.

Reference Standards: In present study 14 standards of phenolic and flavonoid compounds were used including; Epicatechin, Ferulic acid, Hyperoside, Gallic acid, Caffeic acid, Chlorogenic acid, Luteolin, Fisetin, Rutin, Naringenin, Apigenin-7- o-glucoside, Benzene-triol, Chrysin and Apigenin.

Plant Samples: The samples for analysis were crude methanolic extracts (ME) and active fractions of three selected active plants i. e. *G. wallichianum* (GWME, GWEF, GWBF and GWAF), *E. parvifolia* (EPME, EPEF, and EPAF) and *A. alpinum* (AAME, AAEF, AABF and AAAF).

Preparation of Samples: Stock solutions of all samples of standard phenolic compounds and extract samples (1mg/mL stock) were prepared using HPLC grade methanol in amber Eppendorf tubes to protect from light and filtered through nylon membrane filters (0.45 µm).

Methodology for HPLC: Waters Breeze™ HPLC system with a Zorbax SBC-18 (150x3mm, 3.5µm) column and 1525 binary pump was used to analyze the phenolic and

flavonoid compounds in samples where 717 plus auto sampler (injection volume of 10µL), equipped to in-line degasser and 2487 UV detector. Temperature of column oven was set to 48°C and 60°C with flow rate 1 mL/min. Run time was 70 min for each samples and all the detection of each sample were made at 330nm.Gradient elution system of two mobile phases i.e. mobile phase 1(A) was phosphate buffer (pH 2.3, 85% orthophosphoric acid) whereas mobile phase 2 (B) was methanol (HPLC grade) was used. Gradient elution was buffer/methanol 95:5 (0-52 min), 58:42 (52-57 min) then again with initial composition (57-60 min) having flow rate of 1mL/min and was let to run for another 10 minutes before injecting the next sample (Ijaz *et al.*, 2019).

Identification of compounds by HPLC: Compounds were identified by comparing the retention time and absorption spectrum of samples constituents to reference compound used.

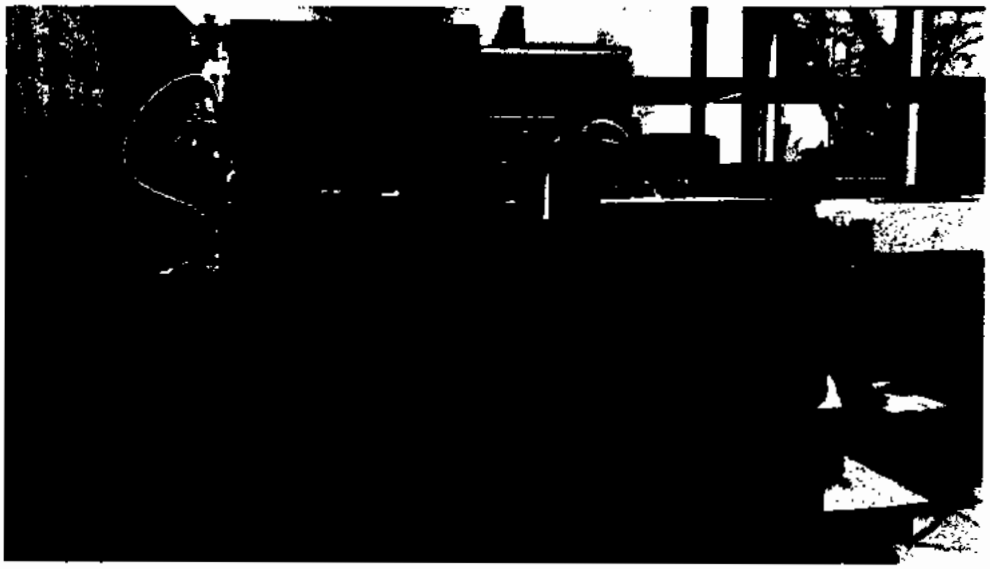


Figure 3.9: HPLC Instrument for Qualitative Analysis of Plants Extracts

Mobile Phase Conditions: Mobile phase gradient elution for HPLC was followed as shown in table 3.2 below.

Table 3.2: Gradient Elution of Mobile Phase Used in HPLC

Time (min)	%A	%B	mL/min
0	95	5	1
52	58	42	1
57	58	42	1
57.1	95	5	1
60	95	5	1

3.14.2. LCMS-Analysis

The methanolic extracts and most active fractions of selected active plants (*G. wallichianum*, *E. parvifolia* and *A. alpinum*) were subjected for LC-MS analysis to identify the active constituents with standard procedure below.

LC-QTOF-MSMethodology:LC-ESI-Q-TOF-MS apparatus composed by Agilent technologies (Santa Clara, USA) was applied in the study. The instrument was made up of HPLC chromatograph (1260 Series) containing a degasser (G1322A), a binary pump (G1312C), an auto sampler (G1329B), a PDA detector (G1315D), column oven (G1316A) and mass spectrometer (G6530B) along with quadrupole and a time-of-flight analyser. Ionization mode, both negative and positive were applied to achieve spectra of all components with high resolution. Acetonitrile (B) and water (A), with the addition of formic acid in both was used as gradient elution; adjusted for sufficient separation of the extract through chromatographic column (Zorbax Stable Bond RP-18 column: 150 mm x 2.1 mm, dp =3.5μm): 0 min–1% B in A, 5 min – 10 % B in A, 20 min – 40 % B in A, 35 min –95% B in A, 36 min –1 % B in A, with stop time: 45 min. 0.2 mL/min flow rate with two (2) min post time, injection volume 10 μL and the range from 100-1200) adjusted in the method. Performance of this analysis was done at 25 ℃ by freshly calibrated apparatus. Fragmentation was simultaneously performed of two most intensive signals in the MS/MS analysis. They were excluded for 0.3 min from the analysis to collect more fragmentation

data after collection of one spectrum from other – less intense signals. A calibration mixture as an internal standard was dosed simultaneously during the analysis to sustain measurement with high accuracy. A calibration curve of quercitrin was obtained from Sigma Aldrich (St. Louis, USA) higher than 95% purity for quantitative analysis of flavonoids. The calibration curve equation (y= 0931215930x +14990549) with R^2 (0.997) value were calculated from the 7 standard's injections at the same injection volume within the range of 0.001-0.04 mg/mL. Validation of majority of MS parameters settings was done initially and afterwards set as following: The capillary voltage 3500 V, fragmentor energy 150 V, temperature of gas 350 °C, sheath gas temperature 325 °C, drying gas flows 12 L/min, nebulizer 35 psig, skimmer of 65 V, collision energies of 10-20 V. The following factors were included in the validation of the best-evaluated method: The limit of detection (LOD) expressed as signal-to-noise (S/N) times 3 (measured in the vicinity of the peak of interest), the limit of quantification (LOQ) – calculated as S/N times 10 and linearity (linear range determination) determined for n=5 peak area measurements. Conduction of analysis and the management of the obtained spectral data was achieved by Mass Hunter B.07.00 software (Kukula-Koch, 2017).

3.15. Statistical Analysis

Data obtained for mice was expressed as mean value ± S.E (n = 6 for each group). One-way analysis of variance (ANOVA) was used for comparison among groups using Statistix 8.1, following the Tukey HSD test (Mekky *et al.*, 2016). The level of significance was set as $P < 0.01$. Excel 2016 was used to construct calibration curves and to calculate mean and standard deviation in DPPH assay and TPC and TFC analysis.

RESULTS

4. RESULTS

4.1. Plants Collection and Identification

Ten selected plants were collected and identified as: *Viola canescens* Wall(family-Violaceae) whole plant (voucher specimen number 040948), *Dryopteris ramosa* C.Hope(family- Dryopteridaceae) whole plant (voucher specimen number 040931), *Geranium wallichianum* D. Don ex sweet (family -Gerinaceae) whole plant (voucher specimen number 040996), *Eleagnus parvifolia* Wall ex. Royle (family- Elaeagnaceae) shoots with fruits (voucher specimen number 040998), *Rosa brunonii* Lindl (family-Rosaceae) floral parts with aerial shoots (voucher specimen number 040988), *Aconogonon alpinium* All Shur(family- Polygonaceae) whole plant (voucher specimen number 040991), *Oxyria digyna* (L) Hill(family-Polygonaceae) whole plant (voucher specimen number 040997), *Fragaria nubicola* Hook (family- Rosaceae) whole plant (voucher specimen number 040943), *Thymus serphyllum* Ronniger(family- Lamiaceae) whole plant (voucher specimen number 040993)and *Primula macrophylla* D.Don (family-Primulaceae) whole plant (voucher specimen number 040995). Identification was based on previous data record, morphological characteristics of leaves and flowers. Confirmation about the names of selected identified plants was performed by record of "The Plant List encyclopedia" and flora of Pakistan.

4.2. Crude Methanolic Extraction and Hepatoprotective Effect of Selected Medicinal Plants

4.2.1. Methanolic Extraction

Mass (g) of crude methanolic extract and yield (%) of selected plants is presented in table 4.1 below. Each plant material in dry powder form (120g each) produced; 11.2g of extract with yield of 9.33% from *V.canescens*.15.82g of extract with 13.18% yield from *D.ramosa*, 15.65g extract of *G. wallichianum* with 13.04% yield, 12.40g extract and 10.33%

yield from *E. parvifolia*, 12.20g extract from *R. brunonii* with 10.16% yield,12.92 g extract from *A. alpinium*, with 10.7% yield,12.90 g extract with 10.75% yield from *Oxyria digyna*, 8.95 g extract from *F. nubicola*with 7.45% yield,10.17 g extract from *T. serphyllum* with 8.47% yield and 13.16 g extract from *P.macrophylla* with10.96% yield. The order of yield of extract (%) is; DRME (13.18 %) > GWME (13.04%) > PMME (10.96 %) > ODME (10.75 %) > AAME (10.70%) > EPME (10.33 %) > RBME (10.16 %) > VCME (9.33 %) > TSME (8.47 %) > FNME (1.45 %).

Table 4.1: Weight of Extract and Yield (%) of Selected Plants

S.NO	Plants	Dry Plant Matter (g)	Methanolic Extract (g)	Yield of Extract (%)
1	*V. canescens*	120	VCME= 11.2	9.33
2	*D. ramosa*	120	DRME= 15.82	13.18
3	*G. wallichianum*	120	GWME= 15.65	13.04
4	*E. parvifolia*	120	EPME= 12.40	10.33
5	*R. brunonii*	120	RBME= 12.20	10.16
6	*A. alpinium*	120	AAME= 12.92	10.7
7	*O. digyna*	120	ODME= 12.90	10.75
8	*F. nubicola*	120	FNME= 8.95	7.45
9	*T. serphyllum*	120	TSME= 10.17	8.47
10	*P. macrophylla*	120	PMME= 13.16	10.96

4.2.2. Acute Oral Toxicity Test

Mice orally administered with doses of different plants extracts (VCME flowers, DRME leaves, GWME roots, EPME fruits, RBME flowers, AAME roots, ODME shoot, FNME whole plant, TSME shoots and PMME whole plant) in respective groups with highest dose of 2000 mg/kg b.w did not show any symptoms of behavior changes during initial 4 hrs

and early 24 hrs observations. There was no behavioral change at the end of observation period (14 days) and no mortality observed in any group during 24 hours and up to 14 days of observations. This shows that oral lethal dose (LD_{50}) of these plants extract is greater than 2000 mg/kg. Low and high dose ($1/10^{th}$ and $1/5^{th}$) of 2000mg/kg was selected for further analysis of selected plants for their hepatoprotective effects.

4.3 Hepatoprotective Activity of Methanolic Extract of Selected Plants

4.3.1 Liver Biochemical Parameters

The results of serum liver markers i.e. ALT, AST, ALP (IU/L) and TB (mg/dL) in animals treated with selected plants extracts are represented in figures (4.1- 4.4) below. The level of ALT in all groups (G-I to G-XXIII) is presented in figure 4.1. According to the results, the value of ALT in CCl_4 toxicant control group (G-II) caused the significant ($P < 0.01$) increase from NC group (G-I). The toxic effect of CCl_4 is significantly ($P < 0.01$) decreased in SD (Silymarin100 mg/kg) treatment (G-III) and with 200 mg/kg/d and 400 mg/kg /d of selected plants extracts (VCME, DRME, GWME, EPME, RBME, AAME, ODME, FNME, TSME and PMME) orally administered in treatment groups (IV-XXIII) respectively as compared to CCl_4 toxicant control group (G-II). AST level in CCl_4 toxicant control group (G-II) significantly ($P < 0.01$) increased from NC group (G-I) after CCl_4 intoxication. The toxic effect of CCl_4 was significantly ($P < 0.01$) decreased with SD (Silymarin 100 mg/kg) treatment (G-III) and with 200 mg/kg and 400 mg/kg of selected plants extracts (VCME, DRME, GWME, EPME, RBME, AAME, ODME, FNME, TSME and PMME) orally administered in treatment groups (IV-XXIII) respectively as represented in figure 4.2. Similarly, the level of ALP was reduced significantly ($P < 0.01$) with SD (Silymarin 100 mg/kg) treatment (G-III) and 200 mg/kg and 400 mg/kg of selected plants extracts (VCME, DRME, GWME, EPME, RBME, AAME, ODME, FNME, TSME and PMME) orally administered in treated groups (IV-XXIII) respectively as represented in figure 4.3. TB (mg/dL) in CCl_4 TC group (G-II) caused the significant ($P < 0.01$) increase from NC group (G-I) and this toxic effect of CCl_4 induction was reduced significantly ($P < 0.01$) with SD (Silymarin 100 mg/kg) treatment (G-III) and 200 mg/kg and 400 mg/kg of selected plants extracts (VCME, DRME, GWME, EPME, RBME, AAME, ODME, FNME,

TSME and PMME) orally administered in treatment groups (IV-XXIII) respectively as compared to CCl_4 TC group (G-II) after CCl_4 intoxication as shown in figure 4.4.

Briefly, livers biochemical markers i.e. ALT, AST, ALP & TB in normal control (G-I), CCl_4 toxicant control (G-II), SD (Silymarin 100 mg/kg) treatment (G-III) and effect of all plant extracts (VCME, DRME, GWME, EPME, RBME, AAME, ODME, FNME, TSME and PMME) with 200 mg/kg/d and 400 mg/kg/d against CCl_4 toxicantion in treatment groups (IV-XXIII) respectively, are collectively represented in table 4.2.The value of ALT, AST, ALP & TB in CCl_4 toxicant control group (G-II) increased to 261.36±5.48, 263.32±6.34, 456.72±5.47 (IU/L) and 1.84±0.05(mg/dL) respectively as compared to NC group (52±3.79, 58±4.02, 132±4.24 and 0.56±0.02) after CCl_4 administration. Level of ALT, AST, ALP and TB significantly ($P < 0.01$) decreased in SD (Silymarin 100 mg/kg) treatment (G-III) to 58.80±5.13, 73.30±5.42, 150.98±6.31 and 0.71±0.03 respectively as compare to CCl_4 toxicant control group (G-II). The low dose (200 mg/kg) treatment of respective plants extracts (VCME, DRME, GWME, EPME, RBME, AAME, ODME, FNME, TSME and PMME) orally administered in groups (IV, VI, VIII, X, XII, XIV, XVI, XVIII, XX and XXII) exhibited the significant ($P < 0.01$) decrease in ALT, AST, ALP and TB after CCl_4 intoxication while the level of these parameters further decreased with treatment of higher dose (400 mg/kg/d) in treated groups (V, VII, IX, XI, XIII, XV, XVII, XIX, XXI and XXIII) with orally administered plants extracts respectively as compared to CCl_4 intoxicant control group (G-II). Over all treatment with high dose (400 mg/kg) of GWME, EPME and AAME in treatment groups (G-IX, XI & XV respectively); highly decreased the levels of ALT, AST, ALP & TB that was increased due to CCl_4 intoxication; showing the hepatoprotective effect most actively against CCl_4 intoxication in mice.

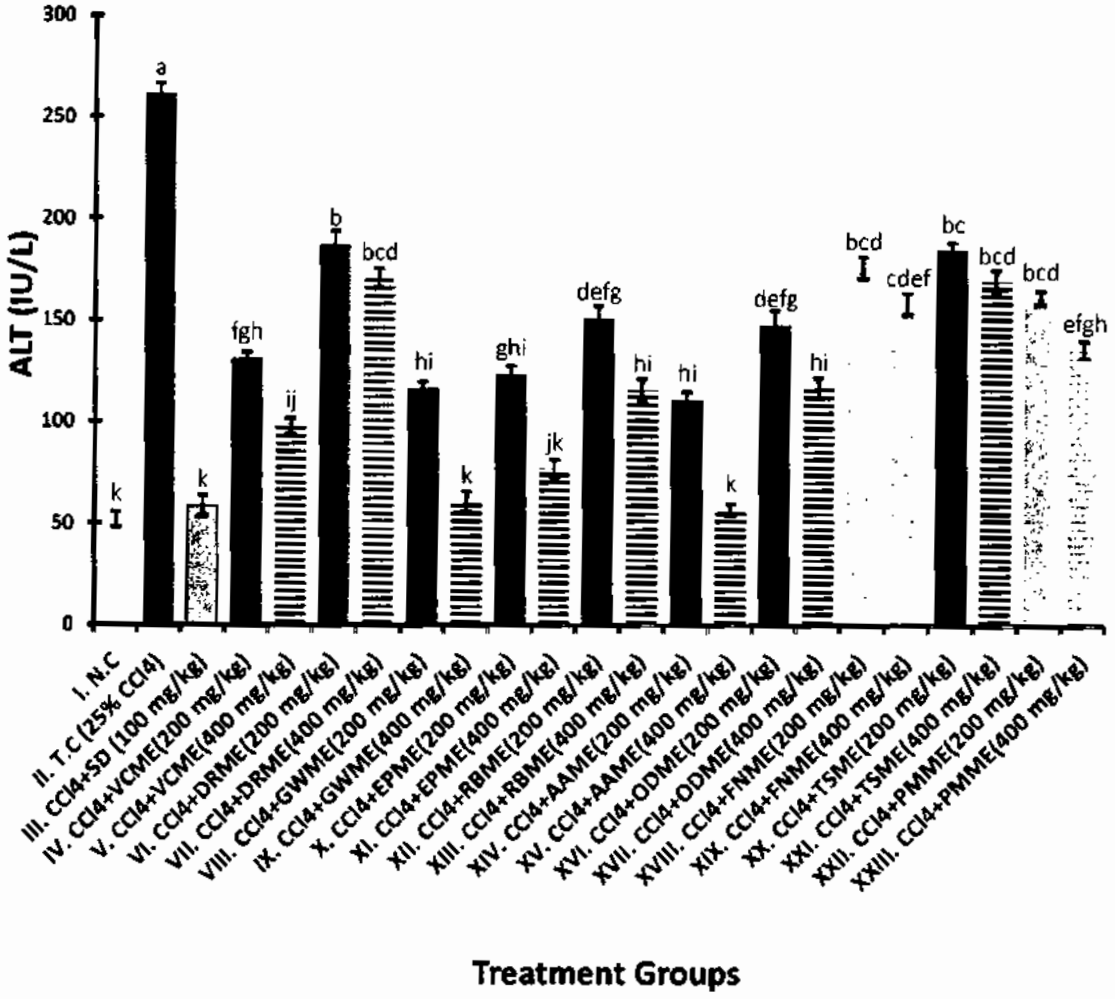
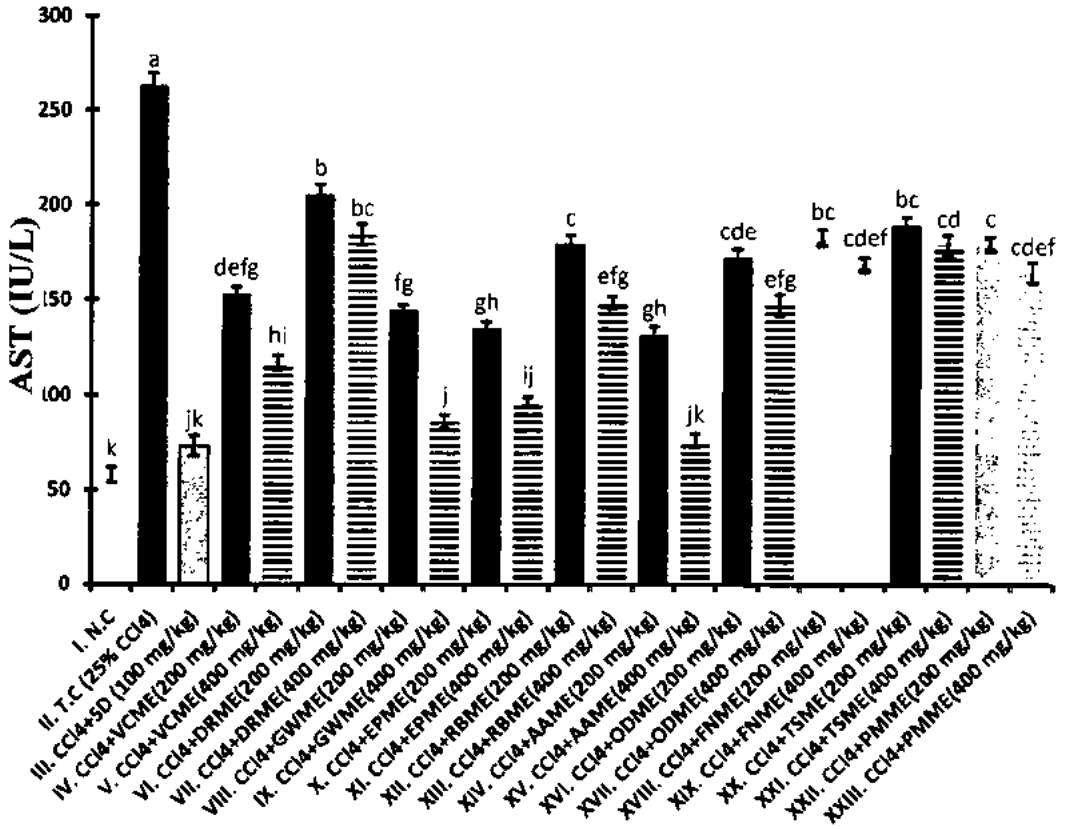


Figure 4.1: Effect of Selected Plants Extracts on ALT in Mice

Results are expressed as mean ± standard error of mean (n= 6). Mean values with different subscripts (a-k) letters are significantly ($P < 0.01$) different from each other while values with same letters are non-significantly ($P > 0.01$) different from each other.



Treatment Groups

Figure 4.2: Effect of Selected Plants Extracts on AST in Mice

Results are expressed as mean ± standard error of mean (n= 6). Mean values with different subscripts (a-k) letters are significantly ($P < 0.01$) different from each other while values with same letters are non-significantly ($P > 0.01$) different from each other.

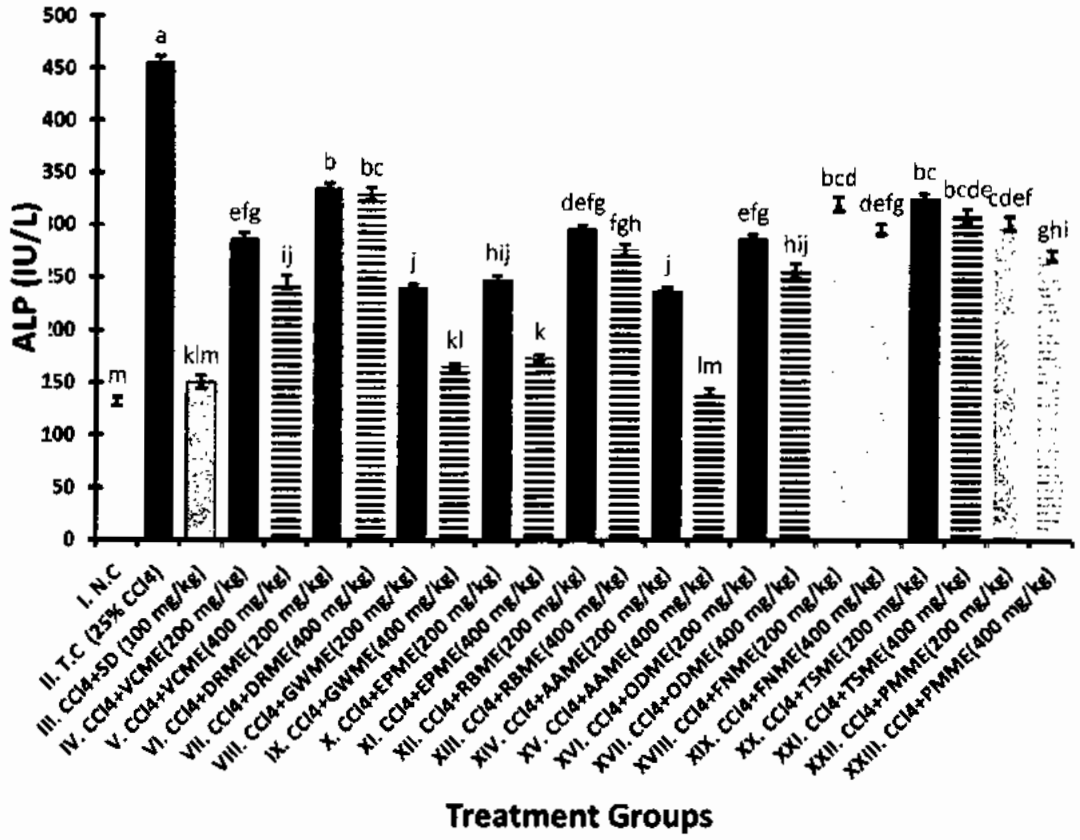


Figure 4.3: Effect of Selected Plants Extracts on ALP in Mice

Results are expressed as mean ± standard error of mean (n= 6). Mean values with different subscripts (a-m) letters are significantly ($P < 0.01$) different from each other while values with same letters are non-significantly ($P > 0.01$) different from each other.

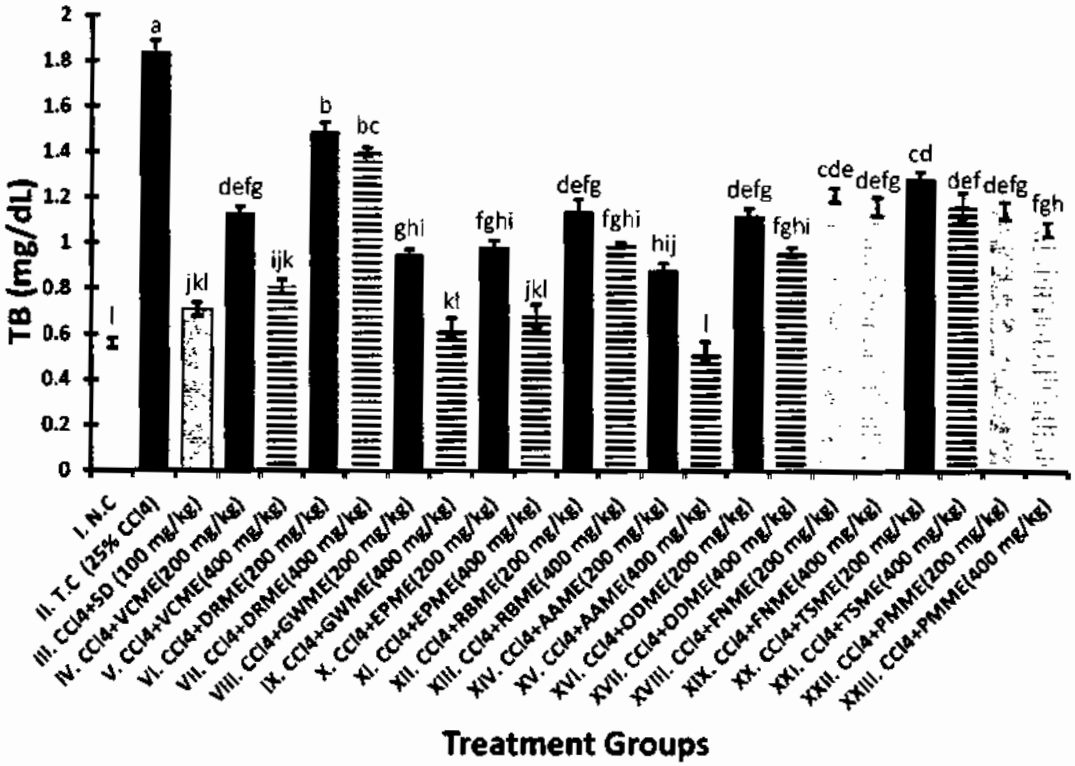


Fig.4.4: Effect of Selected Plants Extracts on TB in Mice

Results are expressed as mean ± standard error of mean (n= 6). Mean values with different subscripts (a-l) letters are significantly ($P < 0.01$) different from each other while values with same letters are non-significantly ($P > 0.01$) different from each other.

Table 4.2: Effect of Selected Plants Extracts on Liver Biochemical Parameters inMice

Group	Treatment	Liver Biochemical Parameters			
		ALT (IU/L)	AST (IU/L)	ALP(IU/L)	TB (mg/dL)
I	NC	52.00 ± 3.79^{k}	58.00 ± 4.02^{k}	132.00 ± 4.24^{m}	0.56 ± 0.02^{l}
II	Toxicant (CCl_4 25%)	261.36 ± 5.48^{a}	263.32 ± 6.34^{a}	456.72 ± 5.47^{a}	1.84 ± 0.05^{a}
III	CCl_4+ SD (100 mg/kg)	58.80 ± 5.13^{k}	73.30 ± 5.42^{jk}	150.98 ± 6.31^{klm}	0.71 ± 0.03^{jkl}
IV	CCl_4+VCME (200 mg /kg)	131.45 ± 2.90^{fgh}	153.17 ± 3.59^{defg}	287.55 ± 5.29^{efg}	1.13 ± 0.03^{defg}
V	CCl_4+VCME (400 mg/kg)	98.00 ± 3.86^{ij}	117.27 ± 3.69^{hi}	246.23 ± 6.47^{ij}	0.81 ± 0.03^{ijk}
VI	CCl_4+DRME (200 mg/kg)	187.30 ± 6.75^{b}	205.74 ± 5.08^{b}	336.03 ± 4.50^{b}	1.49 ± 0.04^{b}
VII	CCl_4+DRME (400 mg/kg)	170.48 ± 4.76^{bcd}	184.39 ± 5.51^{bc}	330.49 ± 5.91^{bc}	1.40 ± 0.02^{bc}
VIII	CCl_4+GWME (200 mg)/kg	116.67 ± 3.21^{hi}	144.85 ± 2.51^{fg}	241.05 ± 2.72^{j}	0.95 ± 0.02^{ghi}
IX	CCl_4+GWME (400 mg/kg)	60.92 ± 4.97^{k}	86.00 ± 3.47^{j}	166.00 ± 2.32^{kl}	0.63 ± 0.04^{kl}
X	CCl_4+EPME (200 mg/kg)	123.40 ± 4.09^{ghi}	135.00 ± 3.51^{gh}	248.30 ± 3.79^{hij}	0.98 ± 0.03^{fghi}
XI	CCl_4+EPME (400 mg/kg)	77.09 ± 4.46^{jk}	96.53 ± 2.22^{ij}	174.13 ± 3^{k}	0.68 ± 0.05^{jkl}
XII	CCl_4+RBME (200 mg/kg)	151.10 ± 5.91^{defg}	179.53 ± 4.34^{c}	297.07 ± 3.46^{defg}	1.14 ± 0.05^{defg}
XIII	CCl_4+RBME (400 mg/kg)	115.84 ± 5.63^{hi}	148.61 ± 3.28^{efg}	277.00 ± 5.48^{fgh}	0.99 ± 0.01^{fghi}
XIV	CCl_4+AAME (200 mg/kg)	111.20 ± 3.72^{hi}	131.51 ± 4.58^{gh}	238.61 ± 3.18^{j}	0.88 ± 0.03^{hij}
XV	CCl_4+AAME (400 mg/kg)	57.06 ± 2.85^{k}	76.05 ± 3.57^{jk}	142.08 ± 3.26^{lm}	0.53 ± 0.04^{l}
XVI	CCl_4+ODME (200 mg/kg)	147.69 ± 6.96^{defg}	172.24 ± 4.22^{cde}	288.07 ± 3.18^{efg}	1.12 ± 0.03^{defg}
XVII	CCl_4+ODME (400 mg/kg)	116.91 ± 4.94^{hi}	147.14 ± 5.81^{efg}	257.76 ± 6.66^{hij}	0.96 ± 0.02^{fghi}
XVIII	CCl_4+FNME (200 mg/kg)	175.81 ± 5.24^{bcd}	182.62 ± 4.14^{bc}	321.45 ± 6.95^{bcd}	1.21 ± 0.03^{cde}
XIX	CCl_4+FNME (400 mg/kg)	158.08 ± 5.37^{cdef}	168.76 ± 3.49^{cdef}	297.13 ± 5.20^{defg}	1.16 ± 0.04^{defg}
XX	CCl_4+TSME (200 mg/kg)	184.15 ± 3.98^{bc}	189.09 ± 4.27^{bc}	326.97 ± 4.36^{bc}	1.28 ± 0.03^{cd}
XXI	CCl_4+TSME (400 mg/kg)	168.94 ± 5.76^{bcd}	178.59 ± 5.21^{cd}	310.57 ± 5.94^{bcde}	1.16 ± 0.06^{def}
XXII	CCl_4+PMME (200 mg/kg)	161.00 ± 3.40^{bcde}	179.09 ± 3.49^{c}	302.68 ± 6.78^{cdef}	1.14 ± 0.04^{defg}
XXIII	CCl_4+PMME (400 mg/kg)	136.08 ± 4.05^{efgh}	164.15 ± 5.3^{cdef}	272.10 ± 5.05^{ghi}	1.06 ± 0.03^{fgh}

Results are expressed as mean ± standard error of mean (n= 6). Mean values with different subscripts (a-m) letters in the column are significantly ($P < 0.01$) different from each other while values with same letters are non-significantly ($P > 0.01$) different from each other.

4.3.2 Percentage (%) Protection of Liver Biochemical Parameters

The percentage (%) protection of liver markers (ALT, AST, ALP & TB) with treatment of SD (silymarin 100 mg/kg) and plants extracts with low dose (200 mg/kg) and high dose (400 mg/kg) against CCl_4 induced toxicity is shown in table 4.3 below. The percentage (%) protection of liver marker enzymes i.e. ALT, AST, ALP (U/L) & TB (mg/dL) with standard drug control (G-III) is 96.75%, 92.53%, 94.15% and 88% respectively. The percentage protection of liver enzymes i.e. ALT, AST, ALP & total bilirubin (TB) with treatment of 200 mg/kg and 400 mg/kg) of VCME in G-IV&V is 62.05%, 53.64%, 52.10% and 55.46% and 78.03%, 71.13%, 64.82% and 80% respectively. The percentage protection of these liver serum parameters/markers enzymes (ALT, & TB in groups (G-VI&VII) treated with DRME with 200 mg/kg &400 mg/kg is 35.37 %, 28.04 %, 37.16 %, and 27.34% and 43.41%,38.44 %, 38.37% and 34.34% respectively which is less than SD treatment (G-III). The percentage protection of these parameters i.e. ALT, AST, ALP & TB in groups (G-VIII & IX) treated with GWME with 200 mg/kg &400 mg/kg is 69.11%, 57.70%, 66.41% and 69% and 95.74%, 86.36%, 89.53%, and 94.53% respectively. The percentage protection of these parameters in groups (G-X & XI) treated with EPME with 200 mg/kg and 400 mg/kg is 61.6 %, 62.50%, 64.18% and 67.18 % and 88.06 %, 81.23 %, 87.02% and 90.62% respectively. The percentage protection of ALT, AST, ALP & TB in groups (G-XII & XIII) treated with RBME with 200 mg/kg &400 mg/kg) is 55.66 %, 40.81 %, 49.16%, and 54.68% and 69.50%, 55.87%, 56.88% and 66.40% respectively. The percentage (%) protection of these liver biochemical parameters in groups (G-XIV & XV) treated with 200 mg/kg and 400 mg/kg) of AAME is 71.12%, 64.20%, 67.16 % and 75% and 97.6%, 91.23%, 96.90% and 102.34% respectively. The percent protection of ALT, AST, ALP & TB in groups (G-XVI & XVII) treated with 200 mg/kg and 400 mg/kg of ODME is 54.30 %, 44.36%, 51.93% and 56.25% and 69 %, 56.58 %, 61.27% and 68.75% respectively. The percentage (%) protection of liver markers i.e. ALT, AST, ALP & TB in groups (G-XVIII & XIX) treated with 200 mg/kg &400 mg/kg of FNME after CCl_4 toxication is 40.86 %, 39.30%, 41.65%, and 49.21% and 49.33%, 46.05%, 49.14% and 53.12% and respectively while in groups (G-XX & XXI)

treated with 200 mg/kg and 400 mg/kg of TSME after CCl_4 intoxication, percentage (%) protection is 36.88 %, 36.15%, 39.95%and 43.75% and 44.14%, 41.26%, 45 % and 53.12% respectively. The percent protection of these liver enzymes (ALT, AST, ALP) & TB in groups (G-XXII & XXIII) treated with 200 mg/kg and 400 mg/kg of PMME after CCl_4 intoxication is 47.93%, 41.02%, 47.43% and 54.68% and 59.84%, 48.30%, 56.85% and 60.93% respectively. The percentage (%) protection of liver enzymes (ALT, AST, ALP (U/L) and TB (mg/dL) with the treatment of extracts (VCME, DRME, GWME, EPME, RBME, AAME, ODME, FNME, TSME and PMME) with 200 mg/kg and 400 mg/kg shows that highest effect is exhibited by the AAME followed by GWME and EPME as compare to other studied plants, represented in table 4.3. The highest percentage (%) protection of ALT, AST, ALP and TB was 97.6%, 91.23 %, 96.90% and 102.34% with 400 mg /kg treatment by AAME (G-XV) followed by GWME (G-IX); 95.74%, 86.36%, 89.53% and 94.53% and EPME (G-XI); 88.06%, 81.23%, 87.02 % and 90.62% which is close agreement with SD (Silymarin 100 mg/kg) treatment (G-I) after CCl_4 induced toxicity as represented below by the table 4.3.

Table 4.3: Percentage (%) Protection of Liver Biochemical Parameters with Treatment of Selected Plants Extracts

Group	Treatment	Liver Biochemical Parameters Percentage (%) Protection			
		ALT (IU/L)	AST(IU/L)	ALP (IU/L)	TB(mg/dL)
III	CCl_4+ SD (100 mg/kg)	96.75	92.53	94.15	88
IV	CCl_4+VCME (200 mg/kg)	62.05	53.64	52.10	55.46
V	CCl_4+VCME (400 mg/kg)	78.03	71.13	64.82	80
VI	CCl_4+DRME (200 mg/kg)	35.37	28.04	37.16	27.34
VII	CCl_4+DRME (400 mg/kg)	43.41	38.44	38.37	34.34
VIII	CCl_4+GWME (200 mg/kg)	69.11	57.70	66.41	69
IX	CCl_4+GWME (400 mg/kg)	95.74	86.36	89.53	94.53
X	CCl_4+EPME (200 mg/kg)	61.6	62.50	64.18	67.18
XI	CCl_4+EPME (400 mg/kg)	88.06	81.23	87.02	90.62
XII	CCl_4+RBME (200 mg/kg)	55.66	40.81	49.16	54.68
XIII	CCl_4+RBME (400 mg/kg)	69.50	55.87	56.88	66.40
XIV	CCl_4+AAME (200 mg/kg)	71.72	64.20	67.16	75
XV	CCl_4+AAME (400 mg/kg)	97.6	91.23	96.90	102.34
XVI	CCl_4+ODME (200 mg/kg)	54.30	44.36	51.93	56.25
XVII	CCl_4+ODME (400 mg/kg)	69	56.58	61.27	68.75
XVIII	CCl_4+FNME (200 mg/kg)	40.86	39.30	41.65	49.21
XIX	CCl_4+FNME (400 mg/kg)	49.33	46.05	49.14	53.12
XX	CCl_4+TSME (200 mg/kg)	36.88	36.15	39.95	43.75
XXI	CCl_4+TSME (400 mg/kg)	44.14	41.26	45	53.12
XXII	CCl_4+PMME (200 mg/kg)	47.93	41.02	47.43	54.68
XXIII	CCl_4+PMME (400 mg/kg)	59.84	48.30	56.85	60.93

4.3.3. Liver Histopathological Analysis on Treatment with ME of Selected Plants

Microscopic histological analysis of liver sections of normal control mice, CCl_4 toxicant control, standard drug treatment (silymarin 100 mg/kg) and selected plant extract (VCME, DRME, GWME, EPME, RBME, AAME, ODME, FNME, TSME and PMME) in treatment groups (G-IV- G-XXIII) after CCl_4 intoxication are shown in figures 4.5-4.7 and 4.8-4.17 (a,b) respectively. Figure 4.5 shows the liver section of normal control (G-I.NC) mice having normal central-vein and well structural intergrity of hepatocytes, kupffer cells and clear sinusoids. Overall there are healthy cells are seen in normal liver of mice. Figure 4.6 of liver section of toxicant control (25% CCl_4) mice havingchange in central-vein (CV), bleeding around portal vein, extensive focal necrosis, inflammatory cells infiltration, sinusoidal dilation (Sd), fatty degeneration (FD) and more ballooning. Liver section of treated mice by SD (100 mg/kg) shows normal central vein, kupffer cell and well-developed hepatocytes (H) clearly separated by sinusoids (SS) as shown in figure 4.7. Figure 4.8 (a, b) shows the treatment effects of VCME with 200 mg/kg and 400 mg/kg after CCl_4 intoxication. There is mild necrosis of cells and fatty degeneration, but hepatocytes and sinusoids are recovered towards normal as shown in figure 4.8 (a) while figure 4.8b shows more improvement in restoration of central vein and maintained arrangement of sinusoids around hepatocytes in dose dependent manner of VCME.Figure 4.9 (a, b) shows mice liver with 200 mg/kg and 400 mg/kg treatment effect of DRME after CCl_4 intoxication in which there is mild sinusoidal dilation, less inflammatory cells infiltration (I) and fatty degeneration (4.9 a) while mild disrupted hepatocytes with more improvement of sinusoids in dose dependent manner of DRME as shown in figure 4.9 b. Figure 4.10 (a, b) indicates the treatment effects of 200 mg/kg and 400 mg/kg) of GWME respectively. There is fatty degeneration of cells but clear central vein and moderate improvement of hepatocytes having sinusoidal separation as shown in figure 4.10 a, while there is high improvement of cellular regeneration having well defined central vein (CV), clear kupffer cells and regular shape of hepatocytes with well-organized sinusoidal spaces as shown in figure 4.10b. Figure 4.11(a and b) shows treatments effects of 200 mg/kg and 400 mg/kg of EPME in which there is clear regular restoration of hepatocytes having sinusoidal spaces and clear central vein (CV) as shown in figure 4.11a while there is more improvement of cellular healing process with well-defined central vein,

sinusoidal spaces separating the organized architecture of hepatocytes and kupffer cells as shown in figure 4.11b. Similarly, figure 4.12a and 4.12b show effects of RBME with its 200 mg/kg and 400 mg/kg treatment after CCl_4 intoxication. There is still dilation of central vein, fatty degeneration and inflammatory cells infiltration and ballooning (4.12a) while some sinusoidal dilation but cellular inflammation with moderate improvement of hepatocytes and clear central vein is seen to reduce (4.12b). Figure 4.13 (a, b) indicates the effects of AAME with 200 mg/kg and 400 mg/kg treatment after CCl_4 intoxication. There are well defined hepatocytes with maintained sinusoidal separations and clear central vein (figure 4.13a) while more improvement of cellular regeneration is observed having clear cellular boundaries with sinusoidal spaces, well organized central vein and albescence of inflammation and ballooning (figure 4.13b). Figure 4.14 (a, b) shows the treatment effects of ODME (200 mg/kg and 400 mg/kg respectively). There is fatty degeneration, focal necrosis and change in architecture of hepatocytes but clear central vein and regeneration ability in sinusoids as shown in figure 4.14 a while mild inflammation only and trend to improvement of cells structure with clear sinusoids is seen in dose dependent manner (figure 4.14 b). In figure 4.15 (a & b), liver sections of mice with treatments effects of FNME with 200 mg/kg and 400 mg/kg by CCl_4 toxicity show that there are mild fatty degeneration or mild ballooning but clear hepatocytes separated with sinusoidal boundaries (4.15a) while more improvement of liver tissue with clear central vein and well organized cellular structure with clear boundaries of sinusoids (4.15b). Figure 4.16 (a, b) of liver section indicates the effects of TSME treatment with 200 mg/kg and 400 mg/kg after CCl_4 intoxication respectively. Much disruption in cells with dilated sinusoids and fatty degeneration (4.16a) but improvement effect on cellular structure is seen by indication of mild fatty degeneration or ballooning, less inflammatory cell and somewhat clear sinusoidal surroundings around hepatocytes (figure 4.16 b). In figure 4.17 (a, b) photographs of liver section with effects of 200mg/kg and 400mg/kg treatment with PMME are shown in which there is hepatoprotective effect, supported by mild cellular changes but clear central vein (CV) and hepatocytes well separated by sinusoids surroundings (4.17a). This effect is further improved by high dose (400 mg/kg) treatment of PMME showing more clear sinusoidal spaces around hepatocytes and well defined central vein as shown in figure 4.17b. Highest improvement of cellular

architecture is seen by treatment effect of AAME followed by GWME and EPME in dose dependent manner after CCl_4 intoxication as compare to treatment effect of other studied selected plants extract.

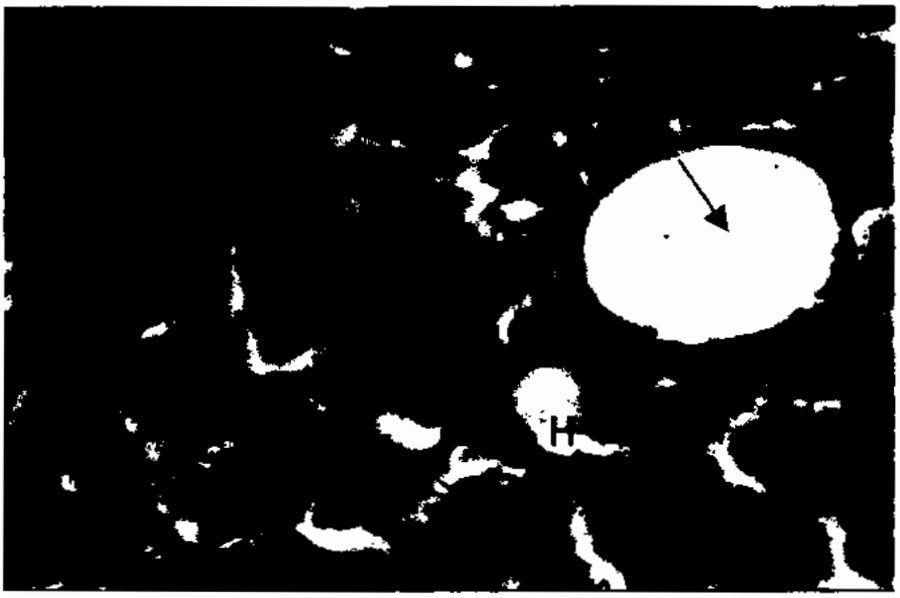


Figure 4.5: Photomicrograph of Liver Section of NC Mice (G-I)

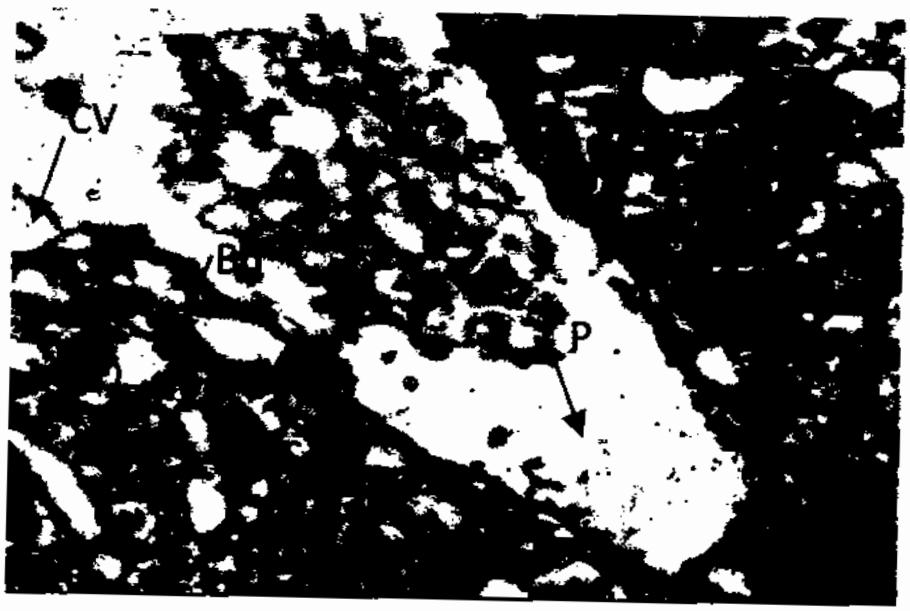


Figure 4.6: Photomicrograph of Liver Section of TC (25% CCl_4) Mice (G-II)

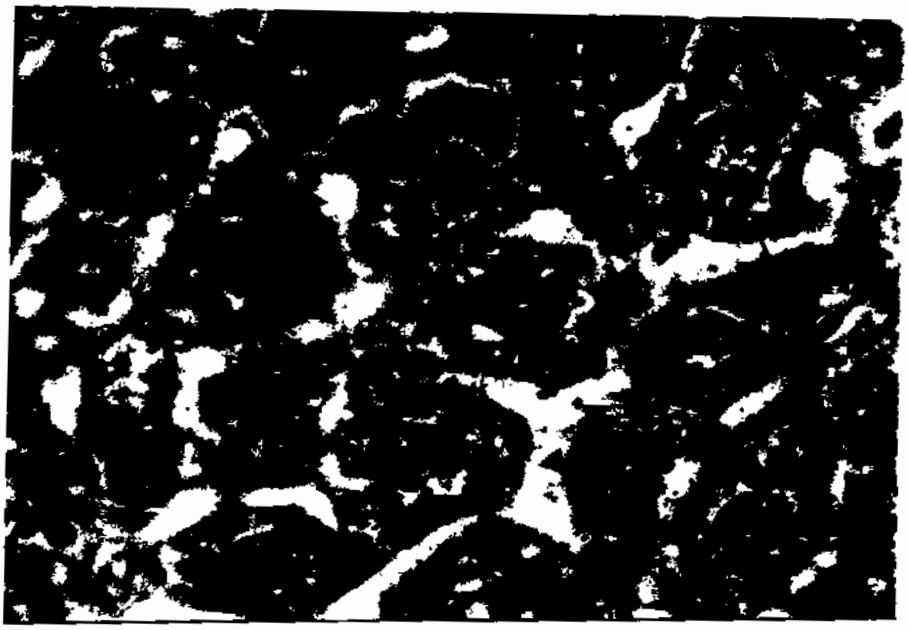


Figure 4.7: Photomicrograph of Liver Section of G-III: CCl_4+SD (100 mg/kg)

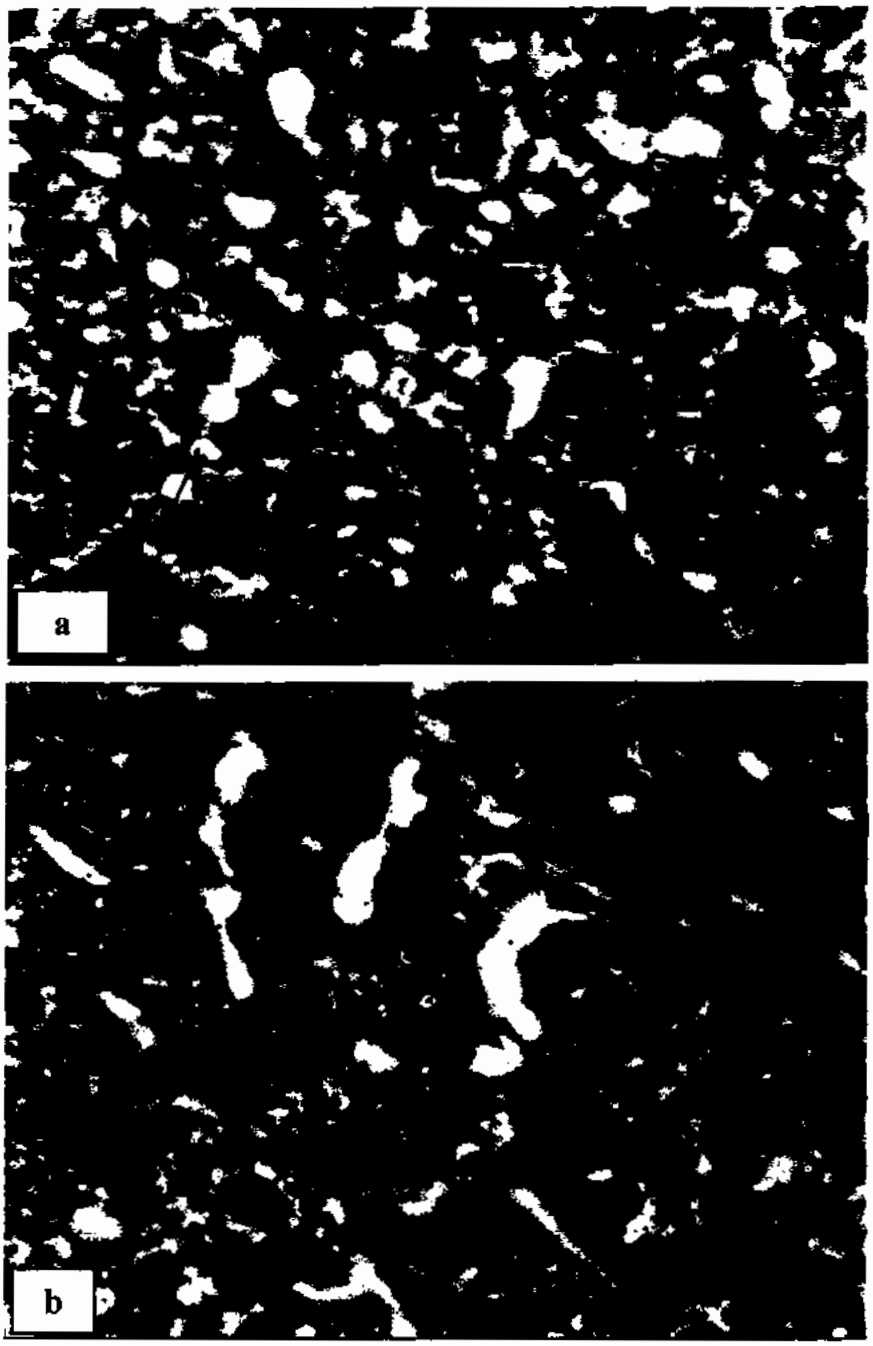


Figure 4.8: Photomicrograph of liver Section G-IV: CCl_4+ VCME 200 mg/kg (a) and G-V: CCl_4+VCME 400 mg/kg (b)

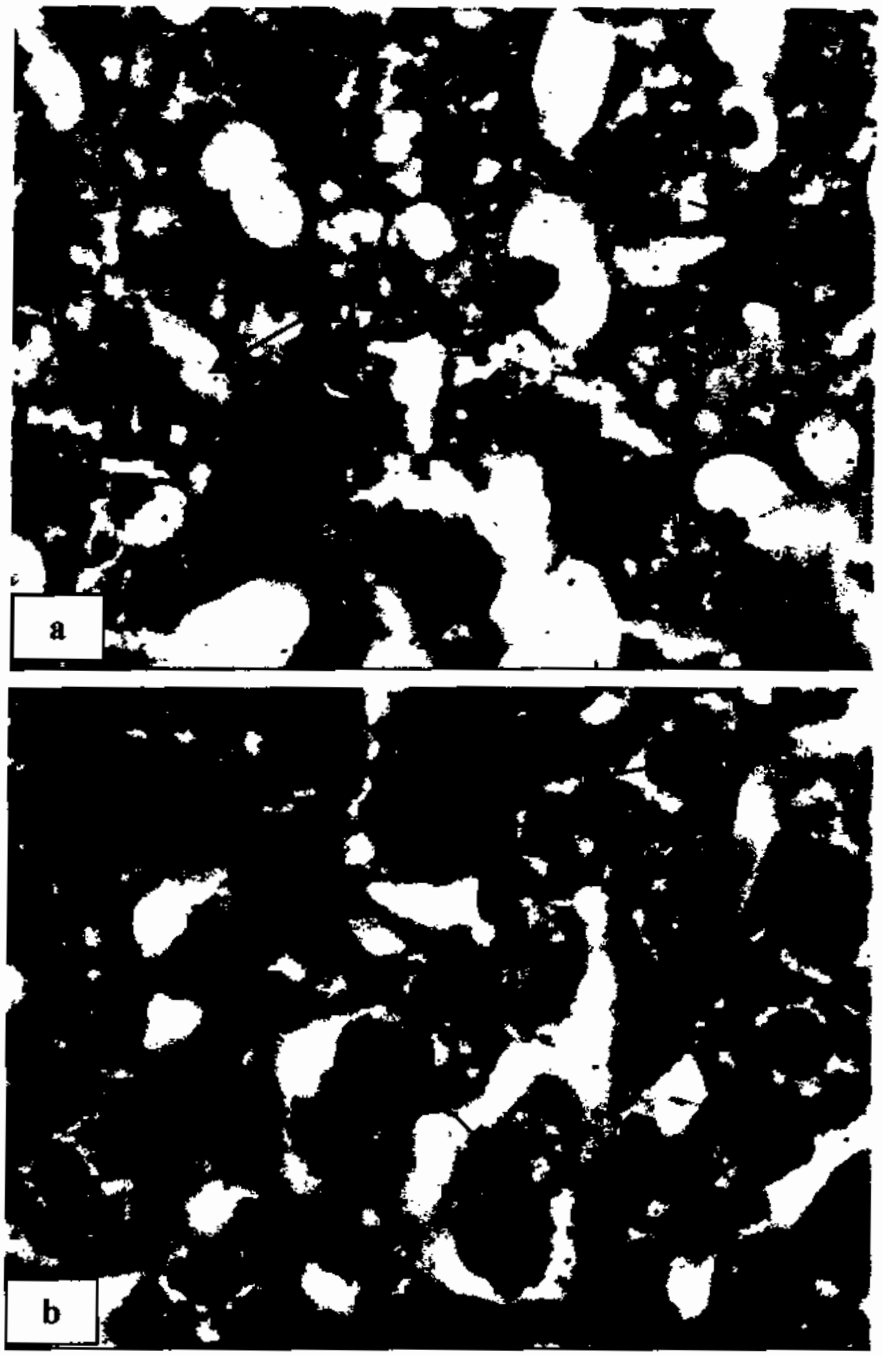


Figure 4.9: Photomicrograph of Liver Section of G-VI: CCl_4+ DRME 200 mg/kg (a) and G-VII: CCl_4+ DRME 400 mg/kg (b)

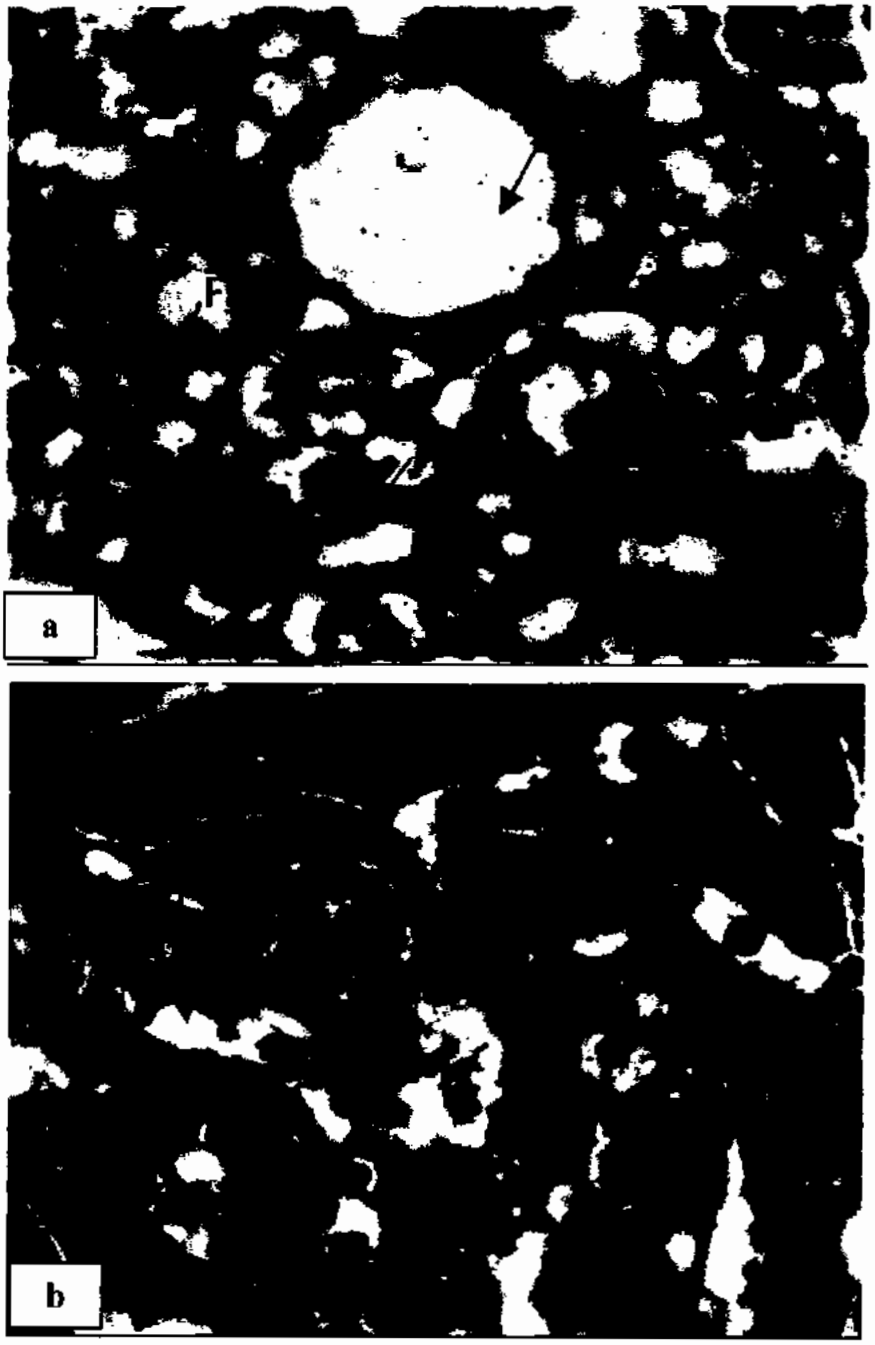


Figure 4.10: Photomicrograph of Liver Section of G-VIII: CCl_4+ GWME 200 mg/kg (a) and G-IX: CCl_4+ GWME 400 mg/kg (b)

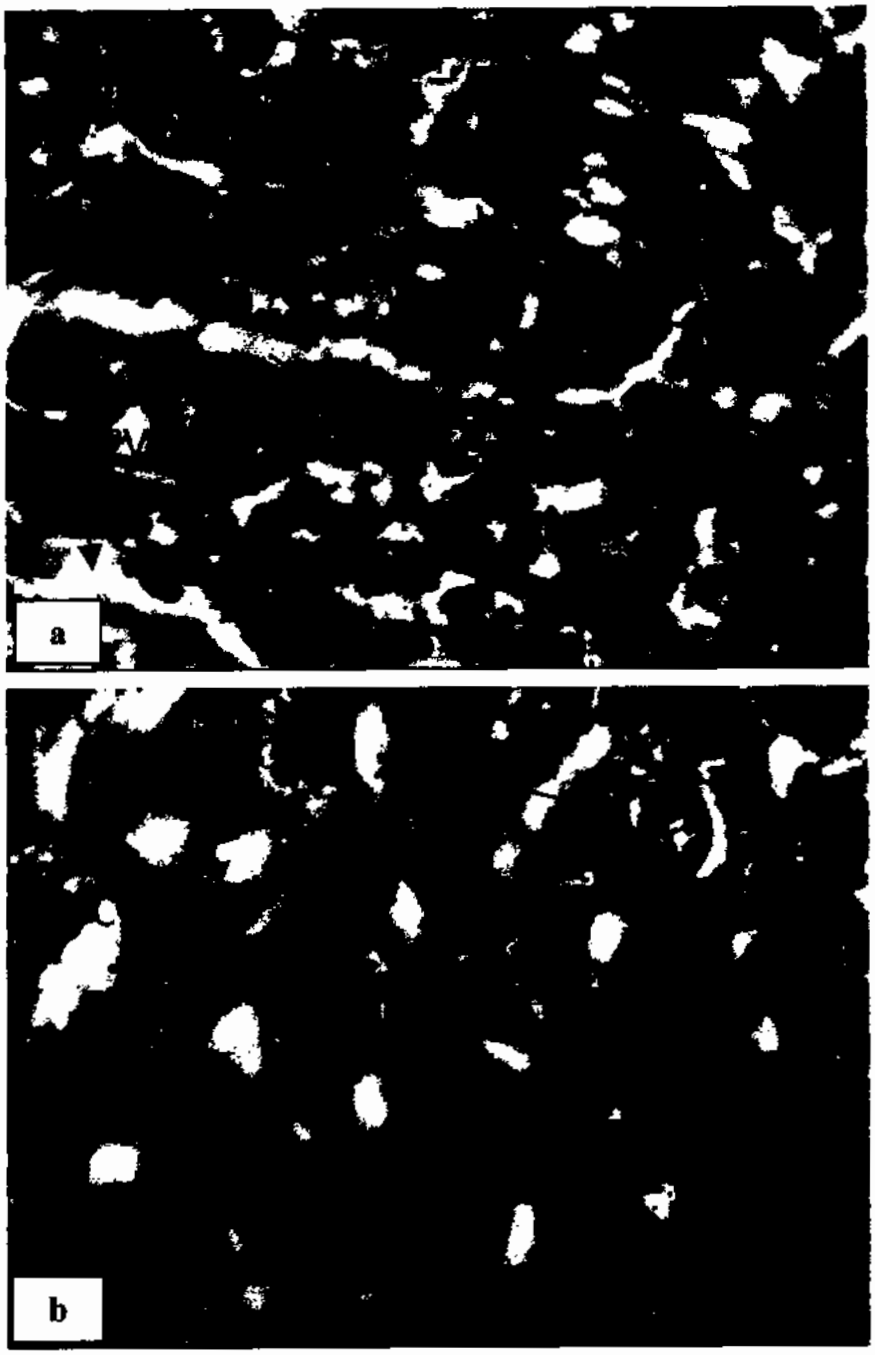


Figure 4.11: Photomicrograph of Liver Section of G-X: CCl_4+ EPME 200 mg/kg (a) and G-XI: CCl_4+ EPME 400 mg/kg (b)

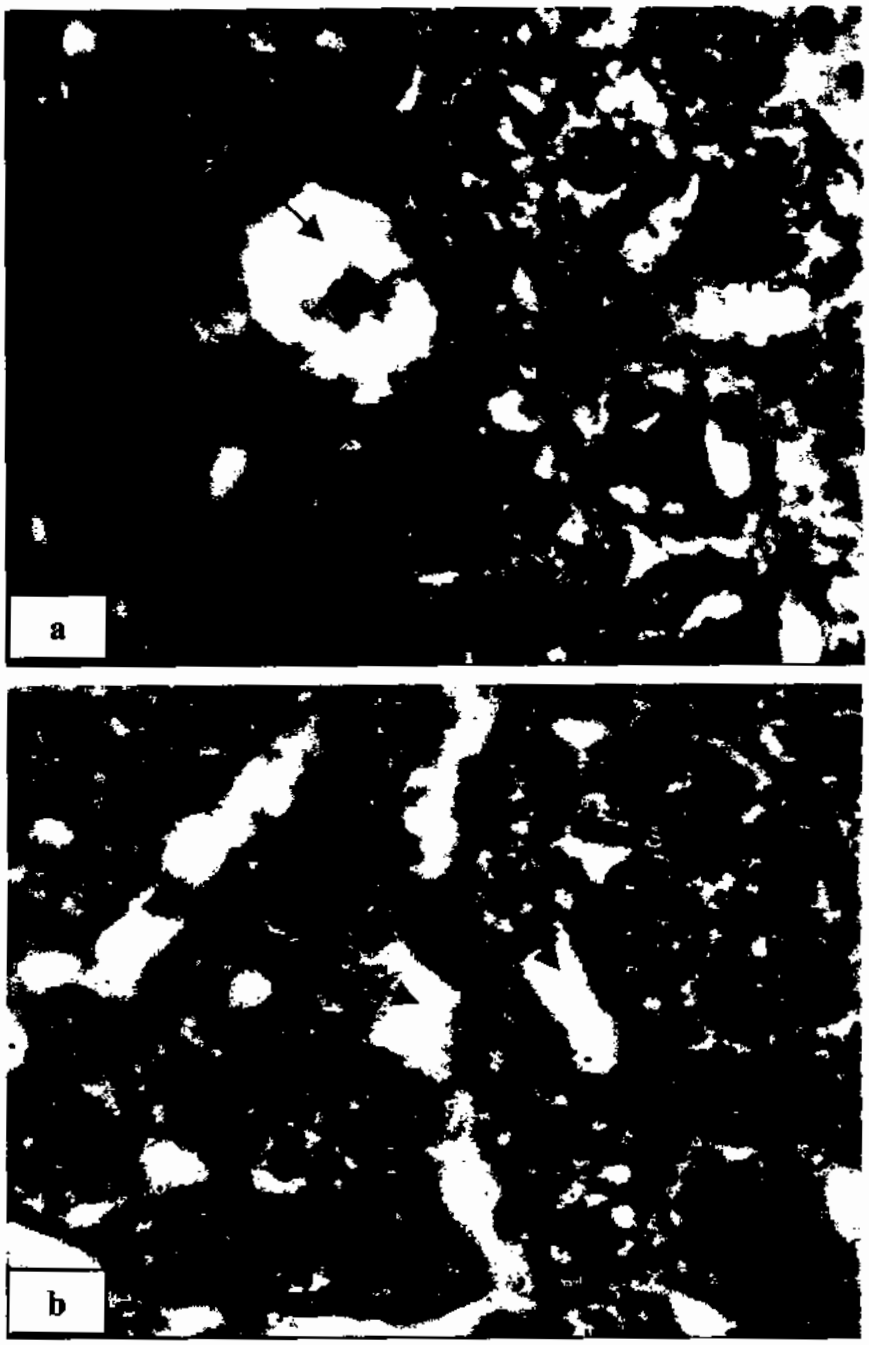


Figure 4.12: Photomicrograph of Liver Section of G-XII: CCl_4+ RBME 200 mg/ kg (a) and G-XIII: CCl_4+ RBME 400 mg/kg (b)

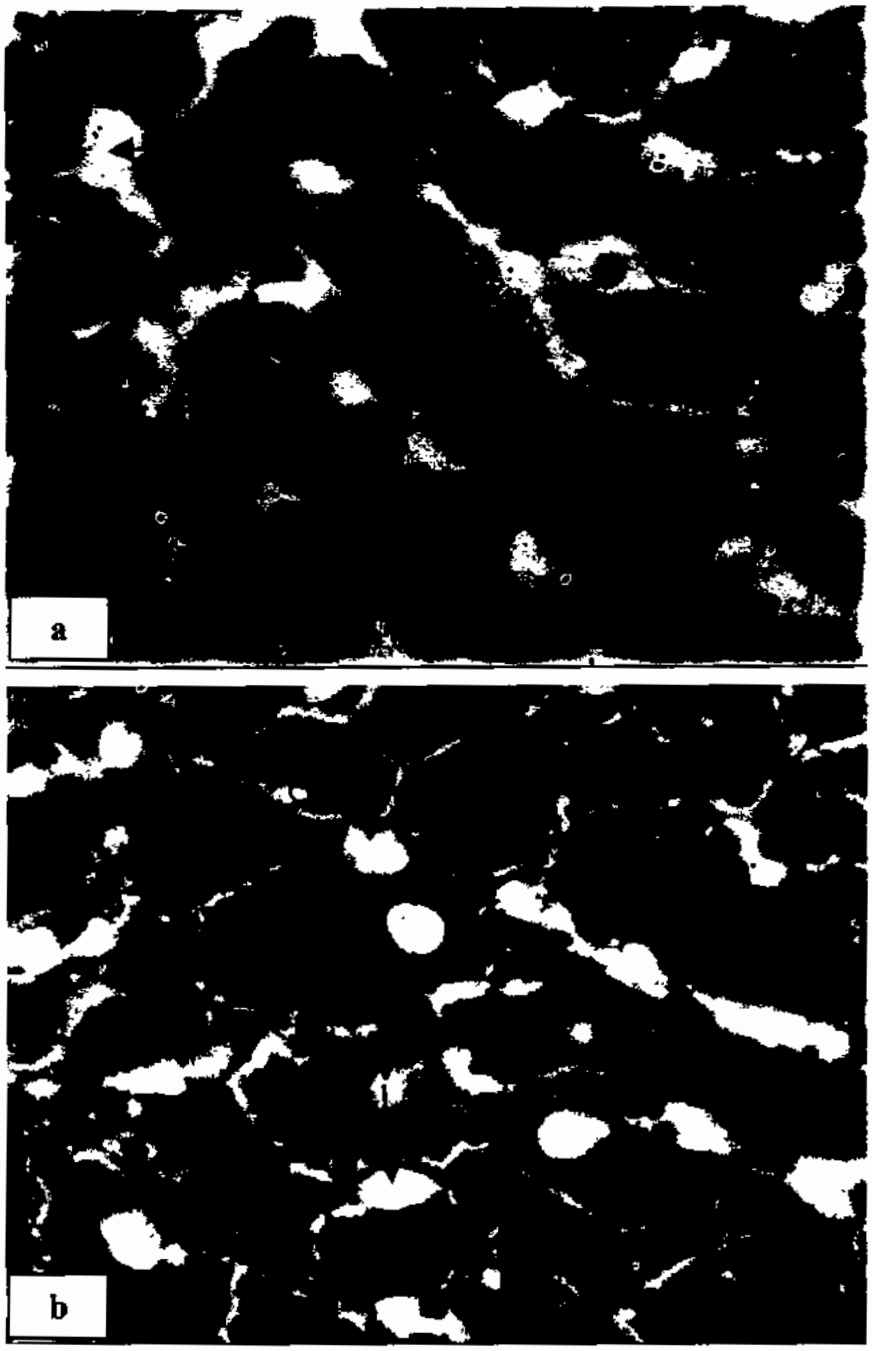


Figure 4.13: Photomicrograph of Liver Section of G-XIV: CCl_4+AAME 200 mg/kg (a) and G-XV: CCl_4+AAME 400 mg/kg (b)

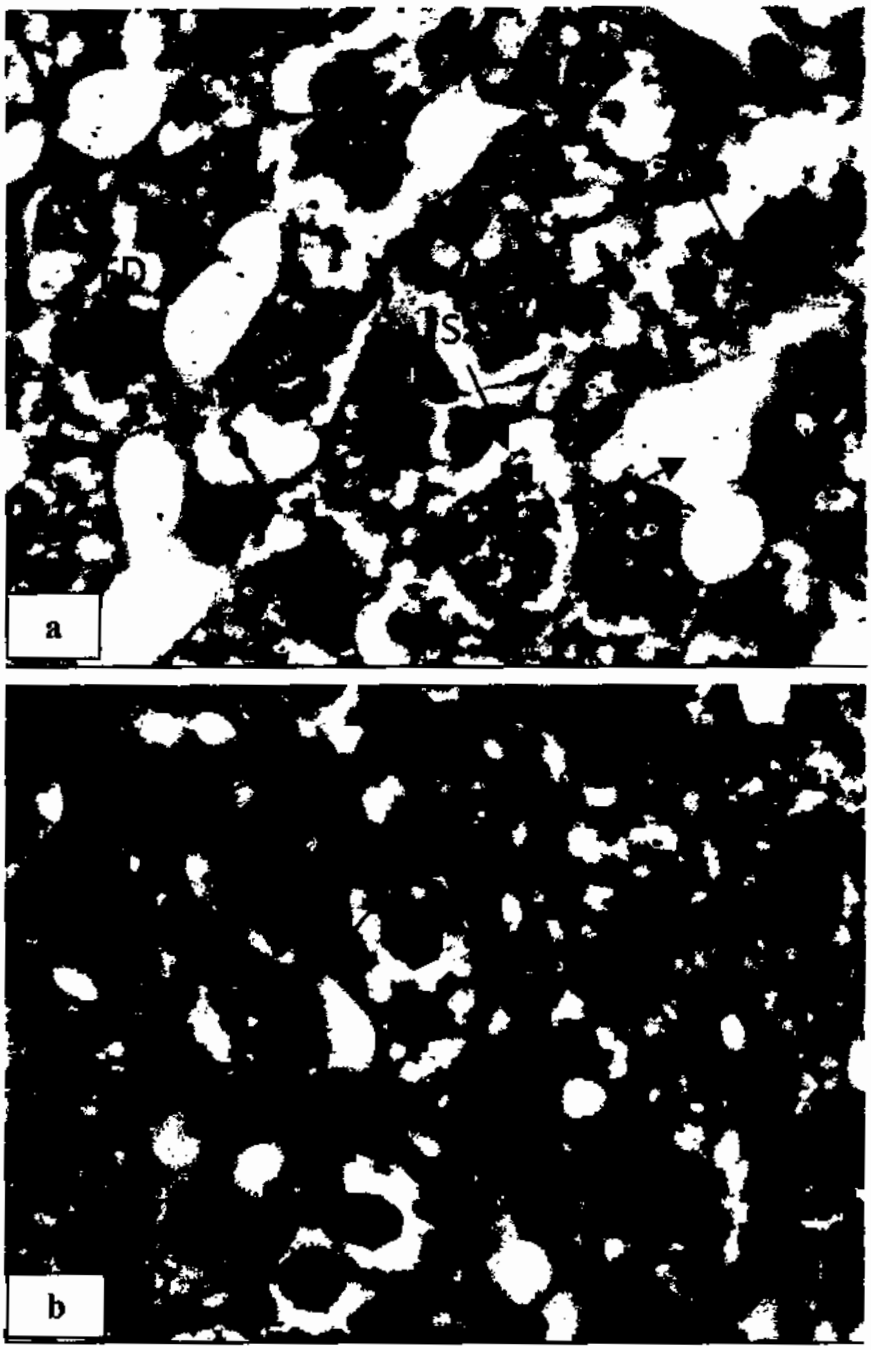


Figure 4.14: Photomicrograph of Liver Section of G-XVI: CCl_4+ODME 200 mg/kg (a) and G-XVII: CCl_4+ODME 400 mg/kg (b)

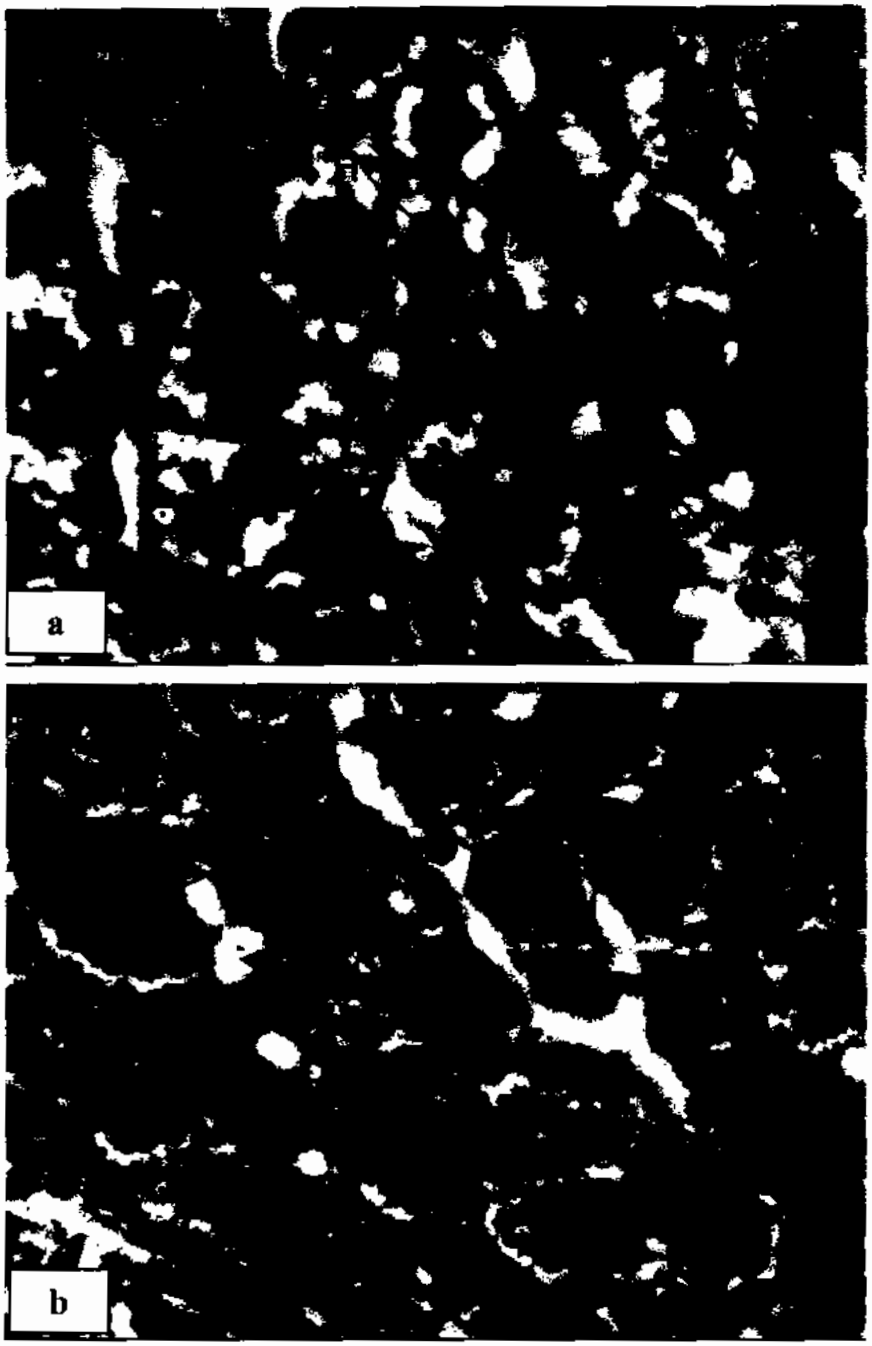


Figure 4.15: Photomicrograph of Liver Section of G-XVIII: CCl_4+ FNME 200 mg/kg (a) and G-XIX: CCl_4+ FNME 400 mg/kg (b)

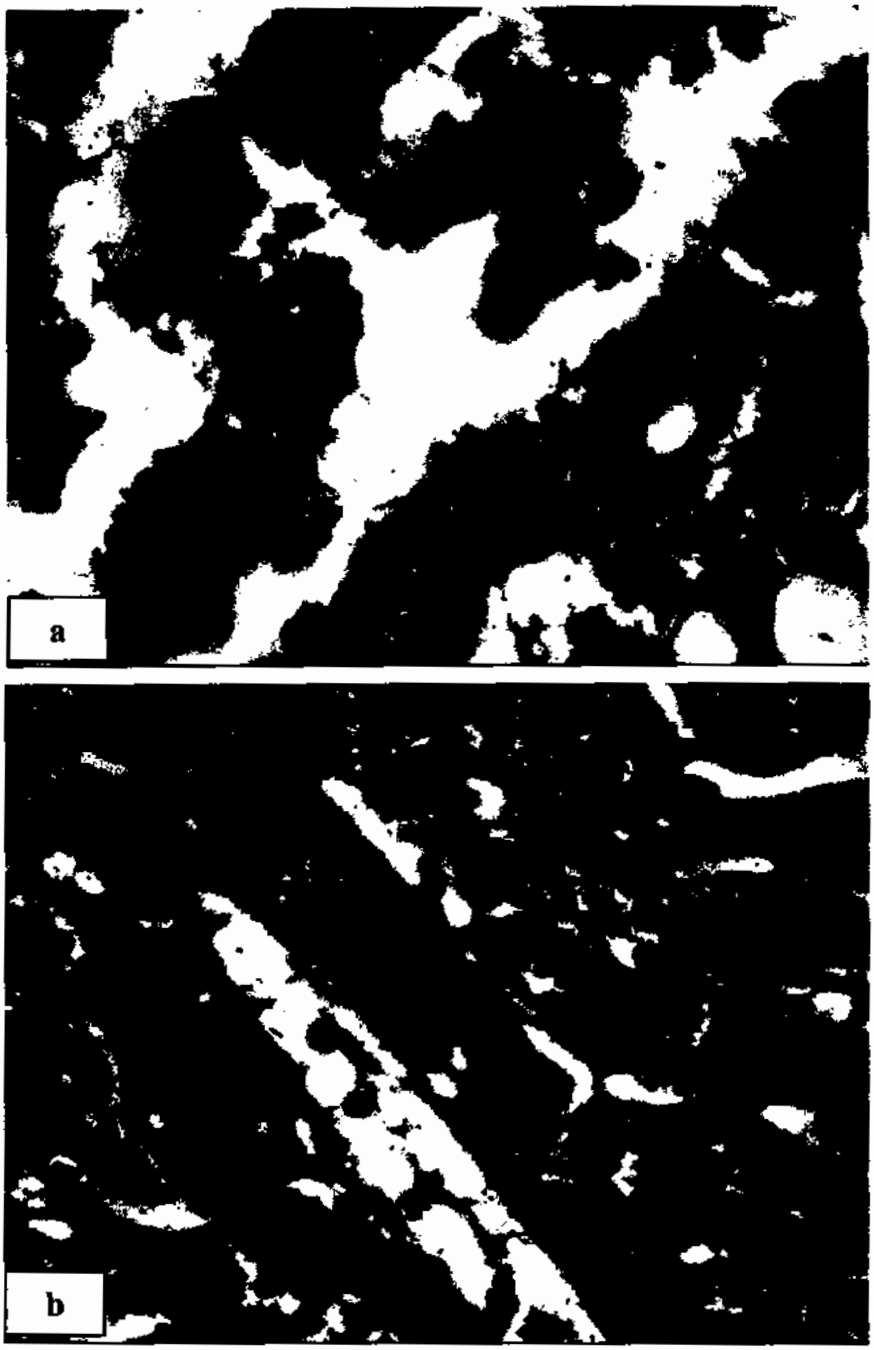


Figure 4.16: Photomicrograph of Liver Section of G-XX: CCl_4+TSME200 mg/kg (a) and G-XXI: CCl_4+TSME 400 mg/kg (b)

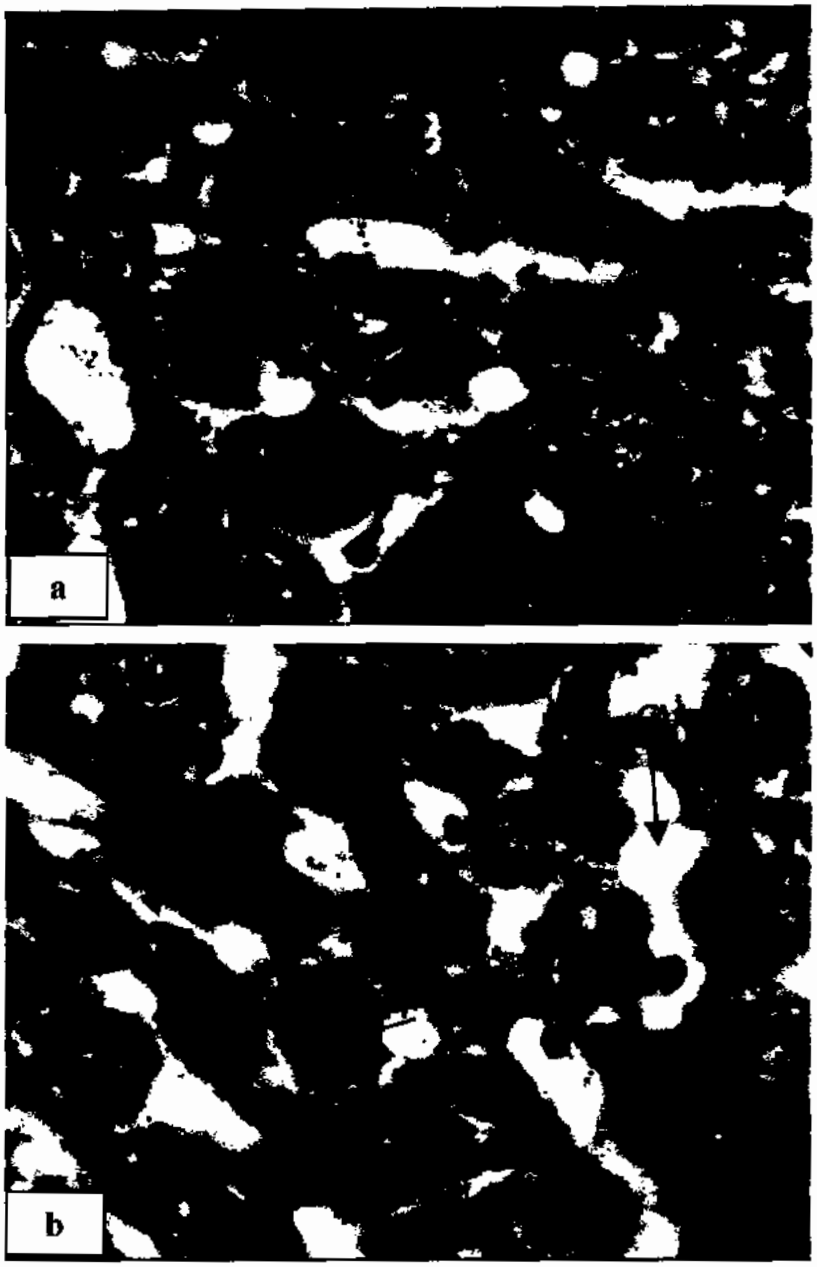


Figure 4.17: Photomicrograph of Liver Section of G-XXII: CCl_4+PMME 200 mg/kg (a) and G-XXIII: CCl_4+PMME 400 mg/kg (b)

H&E magnification100X (figure 4.5- 4.17). Arrows show: CV- ventral vein; SS- sinusoids; H- hepatocytes; Bd-bleeding; FN- focal necrosis; I- inflammatory cells infiltration; P- portal vein; Sd- sinusoidal dilation and FD-fatty degeneration and K- kupffer cell.

4.3.4. Short Listing of Most Active Hepatoprotective Plants

By comparing the results of selected plants extract in treated groups (IV- XXIII) among themselves, compared to NC (G-I) and toxicant control (25% CCl_4 1 mL/kg) in G-II, the order of hepatoprotective effect of these selected plants extract is AAME > GWME > EPME > VCME > ODME > RBME > PMME > FNME > TSME > DRME. The highest percentage (%) protection of ALT, AST, ALP and TB was performed with dose of 400 mg /kg treatment of AAME (G-XV); 97.6%, 91.23 %, 96.90% and 102.34% followed by GWME (G-IX); 95.74%, 86.36%, 89.53% and 94.53% and EPME (G-XI); 88.06%, 81.23%, 87.02% and 90.62 which is close agreement with SD (Silymarin 100 mg/kg) treatment (G-I) after CCl_4 induced toxicity. This shows that AAME, GWME and EPME showed maximum liver protection in mice against carbon tetrachloride induced toxicity. Crude methanolic extracts of *G. wallichianum* roots, *E. parvifolia* fruits and *A. alpinium* roots performed highest decreasing effects in liver markers i.e. ALT, AST, ALP and TB in the dose dependent manner (400 mg/kg) as compare to T.C (CCl_4) group as indicated in tables 4.2 and 4.3.They have highest protective effects on livers architecture (well defined hepatocytes, central vein, sinusoidal spaces, cytoplasm and nuclei) as well as indicated in figures 4.10b, 4.11b and 4.13b which is helpful for decision that these are most active plants for hepatoprotective effect. That is why these three active plants are short-listed for further analysis.

4.4. Extraction and Fractionation of Short Listed Most Active Hepatoprotective Plants

4.4.1. Extraction of Short Listed Most Active Plants

As dry grinded material of each of these three plants used for further studies was 2kg each. They were subjected for further extraction by methanol with same procedure as adopted earlier to get more quantity of their extracts having *G. wallichianum*=260.8 g, *E. parvifolia*=206.6 g and *A. alpinum*=215.2 g as shown in table 4.4 below.

Table 4.4: Weight of Extracts (g) of Short Listed Most Active Hepatoprotective Plants

Plant	Dry Material of plant (g)	Mass of Extract (g)
G. wallichianum	2000	260.8
E. parvifolia	2000	206.6
A. alpinum	2000	215.2

4.4.2 Fractionation of Short Listed Most Active Plants

The results of fractionation of methanolic extract of shortlisted active plants (GWME, EPME and AAME) are given as following.

Fractionation of *G. wallichianum*: Fractionation of *G. wallichianum* methanolic extract (GWME) produced the quantity of each fraction was; *G. wallichianum* n-hexane fraction (GWHF) 14.6 g, *G. wallichianum* chloroform fraction (GWCF) 11.5 g, *G. wallichianum* ethylacetate fraction (GWEF) 31.4 g, *G. wallichianum* n-butanol fraction (GWBF) 21.3 g and *G. wallichianum* aqueous fraction (GWAF) 112 g. The order of yield (g/g weight) of fraction is GWAF (112 g) > GWEF (31.4 g) >*GWBF* (21.3 g) > GWHF (14.6 g) > GWCF (11.5 g) as indicated in table 4.5 below.

Fractionation of *E. parvifolia*: Fractionation of methanolic extract *E. parvifolia* fruits(EPME) produced the quantity of each fraction was, *E. parvifolia* n-hexane fraction (EPHF) 9.8 g, chloroform fraction of *E. parvifolia* (EPCF) 14.45 g, *E. parvifolia* ethylacetate fraction (EPEF) 26 g, *E. parvifolia n*-Butanol fraction of (EPBF) 10.2 g and an aqueous fraction of *E. parvifolia* (EPAF) 98.62 g. The order of yield (g/g) of fractions is EPAF (98.62 g) > EPEF (26 g) > EPCF (14.45 g) > EPBF (10.2 g) > EPHF (9.8 g) as indicated in table 4.5 below.

Fractionation of *A. alpinum*: Fractionation of methanolic extract *A. alpinum* (AAME) produced the quantity of each fraction was; AAHF-11.7 g, AACF- 12.68 g, AAEF- 29 g,

AABF-16 g and AAAF-106 g. Order of yield (g/g) of fractions AAAF (106 g) > AAEF (29 g) > EPBF (16 g)> AACF (12.68 g)> AAHF (11.7 g) as shown in table 4.5 below.

Table 4.5: Weight (g) of Fractions of Short Listed Active Plants

Plant	Crude Extract (g)	Solvents Fractions	Fraction (g)
G. wallichianum	200	n-Hexane fraction (GWHF)	14.6
		Chloroform fraction (GWCF)	11.5
		Ethylacetate fraction (GWEF)	31.4
		n-Butanol fraction (GWBF)	21.3
		Aqueous fraction (GWAF)	112
E. parvifolia	200	n-Hexane fraction (EPHF)	9.8
		Chloroform fraction (EPCF)	14.45
		Ethylacetate fraction (EPEF)	26
		n-Butanol fraction (EPBF)	10.2
		Aqueous fraction (EPAF)	98.62
A. alpinum	200	n-Hexane fraction (AAHF)	11.7
		Chloroform fraction (AACF)	12.68
		Ethylacetate fraction (AAEF)	29
		n-Butanol fraction (AABF)	16
		Aqueous fraction (AAAF)	106

4.5. Hepatoprotective Effect of Different Fractions of *G. wallichianum*, *E. parvifolia* and *A. alpinum*

The value of ALT, AST, ALP and TB results of T.C (CCl_4) in G-II increased (207.67±4.51, 212.07±3.76, 417.67±5.60 and 2.38±0.19) from normal control (53.17±5. 38, 63.08±4.98, 171.33±5.38 and 0.66±0.05) group (G-I). These values decreased to 62.05±5.16, 77.03±3.56, 191.17±4.99 and 0.77±0.05 respectively) with the treatment of SD (Silymarin 100 mg/kg) as compare to TC (CCl_4) group (G-II) which are common control groups for treatment effect of different fractions of *G. wallichianum*, *E. parvifolia* and *A. alpinum* as shown in figures (4.18-4.21, 4.30-4.33, 4.39-4.420) below respectively. The results of treatment effects of different fractions of more active plants (*G. wallichianum*, *E. parvifolia* and *A. alpinum*) by CCl_4 induced hepatotoxicity in mice are discussed below as following.

4.5.1. Hepatoprotective Effect of Different Fractions of *G. wallichianum*

4.5.1.1. Liver Biochemical Parameters

The results of ALT, AST, ALP and TB, with effects of 200 mg/kg and 400 mg/kg of different solvent fractions of GW (GWHF, GWCF, GWEF, GWBF and GWAF); orally administered in mice in treatment groups (VA-XIIIA) against CCl_4 induced toxicity are represented in figures 4.18- 4.21 respectively. Results show that 200 mg/kg dose of GW fractions (GWHF, GWCF, GWEF, GWBF and GWAF), orally administered in treatment groups (IVA, VIA, VIIIA, XA and XIIA) after CCl_4 intoxication, the values of ALT significantly ($P < 0.01$) decreased (168.13±3.15, 164.03±4.93, 119±4.60, 125.09±3.65 and 97.04±3.49) respectively as compare to TC (CCl_4) group (G-II). The ALT values further improved to decrease (160.07±3.53, 154.17±4.31, 77.82±3.86, 99.13±4 and 64.10±3.91) with 400 mg/kg dose of fractions of GW (GWHF, GWCF, GWEF, GWBF and GWAF), orally administered in treatment groups (VA, VIIA, IXA, XIA and XIIIA) respectively from CCl_4 toxicant control group (G-II). There is significant decrease ($P < 0.01$) of ALT values with 200 mg/kg and 400 mg/kg of GWEF, GWBF and GWAF as compare to T.C group (G-II), as shown in figure 4.18. After oral administration of 200 mg/kg dose of different fractions ofGW (GWHF, GWCF, GWEF, GWBF and GWAF), in treatment groups (IVA, VIA, VIIIA,

XA and XIIA) after CCl_4 intoxication; the values of AST significantly ($P < 0.01$) decreased (179.98±3.34, 178.05±5.08, 133.03±3.36, 136.17±3.73 and 113±4.08) respectively from CCl_4 toxicant control group (G-II) which further decreased (167.09±5.89, 165.50±3.25, 97.02±4.32, 110±3.65 and 84.08±4.11) with 400 mg/kg doses of GW fractions (GWHF, GWCF, GWEF, GWBF and GWAF), orally administered in treatment groups (VA, VIIA, IXA, XIA and XIIIA) respectively as compared to CCl_4 toxicant control group (G-II). It is shown that there is non-significant difference ($P > 0.01$) by treatment of GWHF and GWCF when compared between themselves while GWEF and GWAF have significantly ($P < 0.01$) reduced the AST level in dose dependent manner, which is comparable to SD treatment (Silymarin 100 mg/kg) group (G-III) as indicated in figure 4.19. On oral administration of 200 mg/kg doses of GW fractions (GWHF, GWCF, GWEF, GWBF and GWAF) in treatment groups (IVA, VIA, VIIIA, XA and XIIA) respectively after CCl_4 intoxication; the values of ALP significantly ($P < 0.01$) decreased (366.05±6.51, 365.04±5.78, 256.33±3.39, 279.06±2.65 and 226±4.04) respectively as compare to T.C group (CCl_4). ALP values further decreased (208.04±4.44, 242.03±4.55, and 190±4.99) in significant ways ($P <0.01$) due to 400 mg/kg doses effects of GWEF, GWBF and GWAF, orally administered in treatment groups (IXA, XI and XIIIA respectively) as compare to T.C (CCl_4) group (G-II). There is non-significant effect ($P > 0.01$) of GWHF and GWCF (356.18±6.60, 350±5.22) with high dose (400 mg/kg), orally administered in treatment groups (VA, VIIA) as compared to other fractions dose (figure 4.20). The values of TB significantly ($P < 0.01$) decreased (1.92±0.09, 1.88±0.03, 1.12±0.06, 1.20±0.03 and 1.08±0.03) as compare to CCl_4 toxicant control group (G-II) on oral administration of 200 mg/kg doses of GW fractions (GWHF, GWCF, GWEF, GWBF and GWAF) in treatment groups (IVA, VIA, VIIIA, XA and XIIA) respectively after CCl_4 intoxication. The values of TB further decreased (0.83±0.05, 0.99±0.06 and 0.70±0.04) with significant ($P < 0.01$) effects with 400 mg/kg) doses of different fractions of GW (GWEF, GWBF and GWAF), orally administered in treatment groups (IXA, XIA and XIII-A) while non-significant ($P > 0.01$) decrease (1.82±0.08, 1.175±0.02) on treatment with 400 mg/kg dose of GWHF and GWCF as compared between themselves with other fractions, as indicated in figure 4.21.

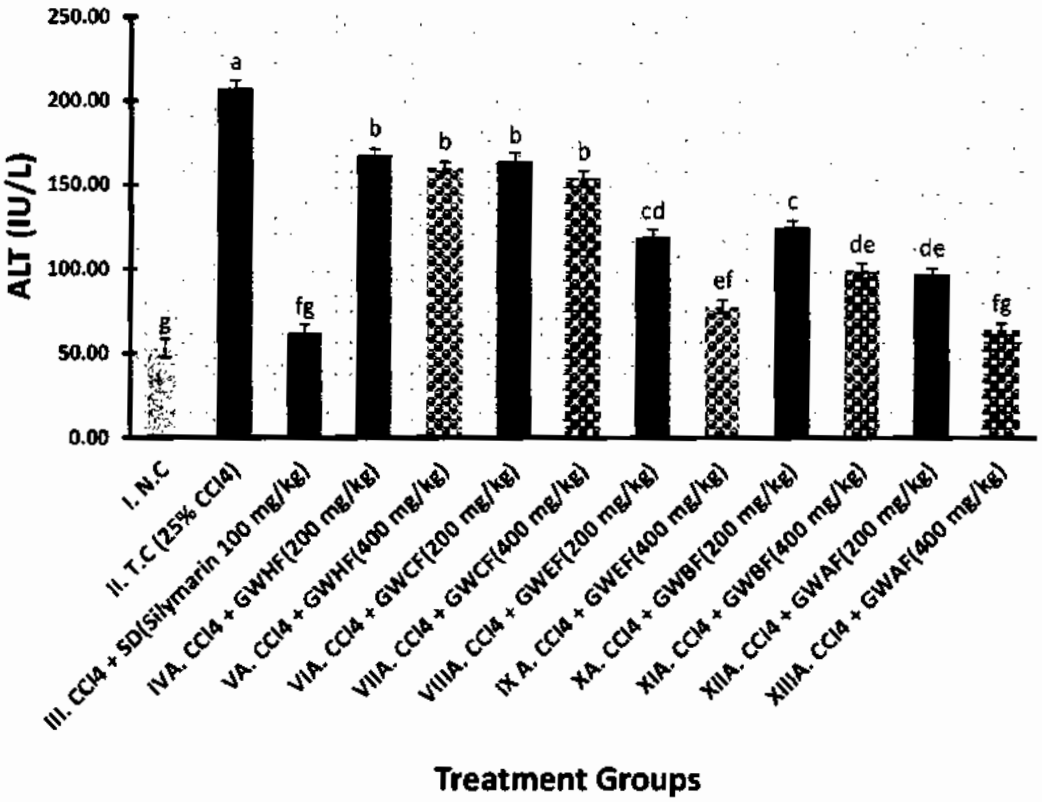


Figure 4.18: Effect of Different Fractions of *G. wallichinaum* (GWHF, GWCF, GWEF, GWBF and GWAF) on ALT in Mice

Results are expressed as mean ± standard error of mean (n=6). Mean values with different subscripts (a-g) letters are significantly ($P < 0.01$) different from each other while values with same letters are non-significantly ($P > 0.01$) different from each other.

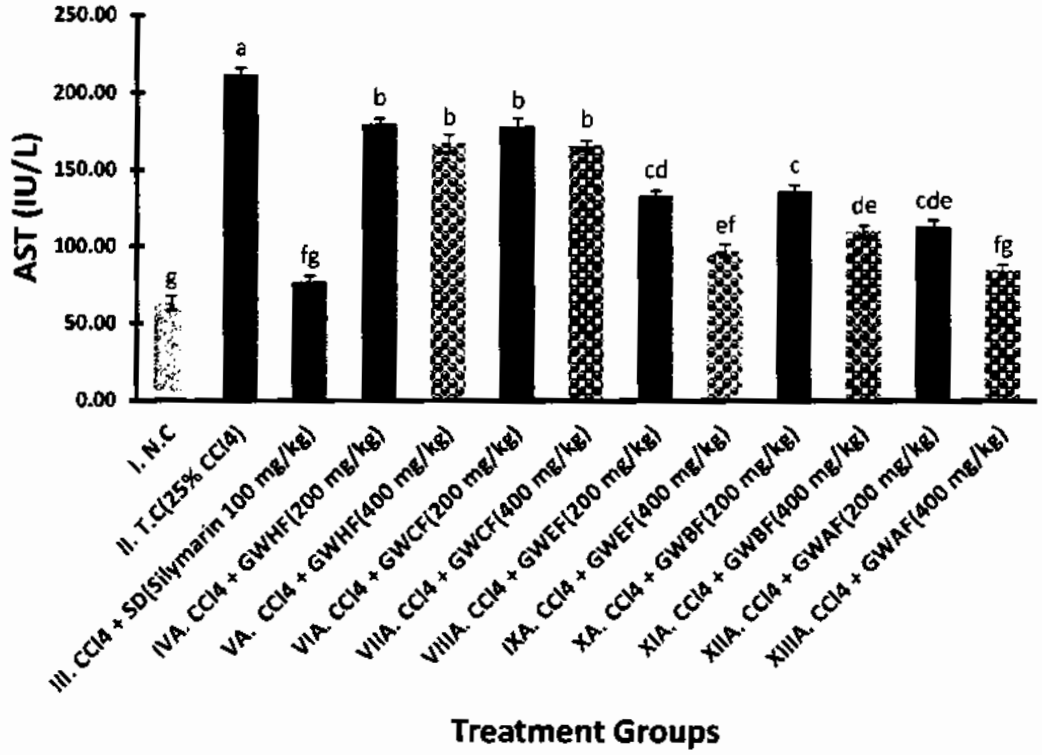


Figure 4.19: Effect of Different Fractions of *G. wallichinaum* (GWHF, GWCF, GWEF, GWBF and GWAF) on AST in Mice

Results are expressed as mean ± standard error of mean (n=6). Mean values with different subscripts (a-g) letters are significantly ($P < 0.01$) different from each other while values with same letters are non-significantly ($P > 0.01$) different from each other.

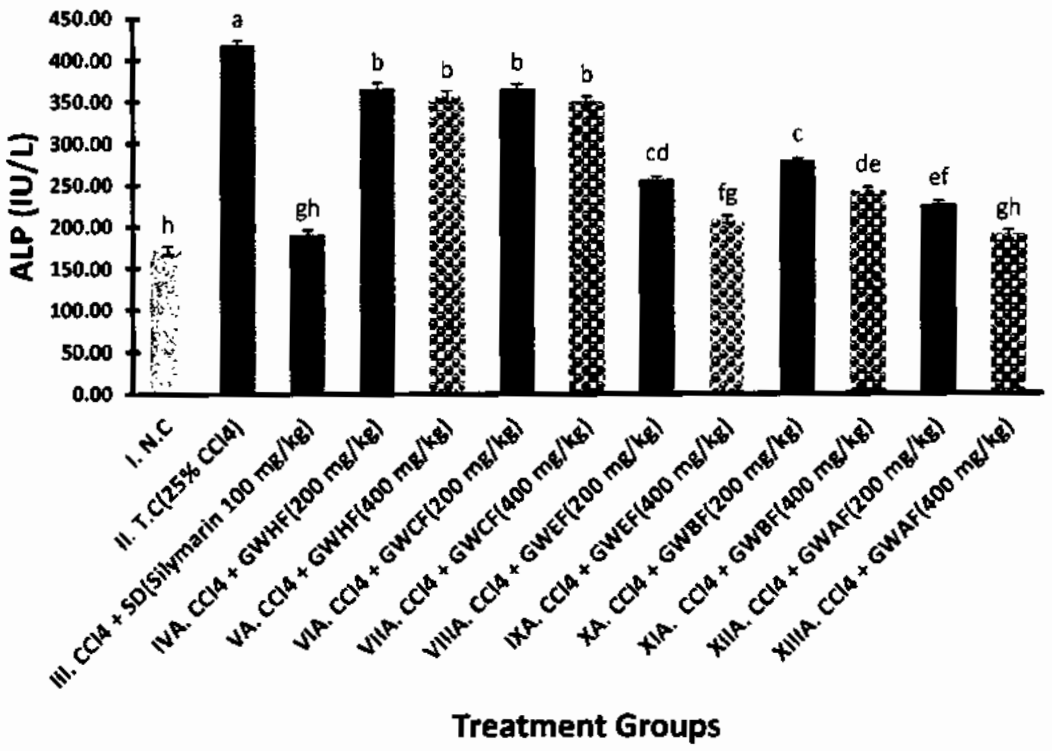


Figure 4.20: Effect Different Fractions of *G. wallichinaum* (GWHF, GWCF, GWEF, GWBF and GWAF) on ALP in Mice

Results are expressed as mean ± standard error of mean (n=6). Mean values with different subscripts (a-h) letters are significantly ($P < 0.01$) different from each other while values with same letters are non-significantly ($P > 0.01$) different from each other.

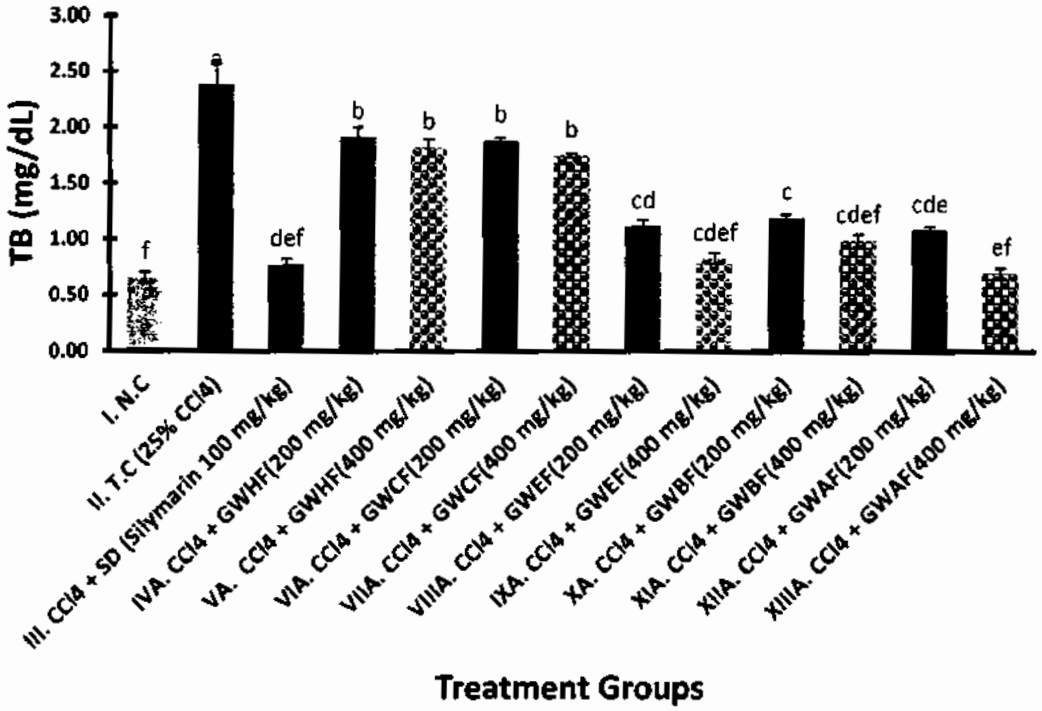


Figure 4.21: Effect of Different Fractions of *G. wallichinaum* (GWHF, GWCF, GWEF, GWBF and GWAF) on TB in Mice

Results are expressed as mean ± standard error of mean (n=6). Mean values with different subscripts (a-f) letters are significantly ($P < 0.01$) different from each other while values with same letters are non-significantly ($P > 0.01$) different from each other.

4.5.1.2. Liver Histopathology on Treatment of *GW* Fractions

Results of microscopic studies of liver histopathology of normal control mice (G-I), CCl_4 toxicant control (G-II) and SD treatment (G-II) are shown in figures 4.22- 4.24. The results of histopathological changes due to effect of fractions of *G. wallichianum* (GWHE, GWCF, GWEF, GWBF and GWAF) on liver sections in treatment groups (IVA- XIIIA) by 200 mg/kg and 400 mg/kg doses after CCl_4 toxicity are shown in figures 4.25 - 4.29 (a, b).

Figure 4.22 shows normal cellular architecture with well-defined hepatocytes and kupffer cells. There are normal nuclei and apparent central vein with clearly separated sinusoidal spaces. Overall, healthy normal cells are seen in liver section. Figure 4.23 reveals that there is bleeding around portal region and deformed central vein. There is fatty degeneration, sinusoidal dilation and severe inflammatory cells infiltration. Cellular structure of hepatocytes is damaged due to focal necrosis. Figure 4.24 shows clear central vein and well-defined structure of hepatocytes clearly separated by sinusoidal spaces. There is clear appearance of kupffer cells that show protective effect of standard drug against CCl_4 induced toxicity. Figure 4.25 (a, b) indicates the treatments effects of GWHF with 200 mg/kg and 400 mg/kg against CCl_4 intoxication. Deformed central vein (CV), sinusoidal dilation and inflammatory cells are there. There is no clear appearance of cellular structure (4.25a). Figure 4.25 (b) shows the clear appearance of central vein (CV), mild necrosis and sinusoidal dilation which reveals that this fraction has less protective effect against CCl_4 induced toxicity. Figure 4.26 (a, b) indicates the treatments effects of GWCF with 200 mg/kg and 400 mg/kg after CCl_4 intoxication, showing fatty degeneration and ballooning, focal necrosis of hepatocytes and loss of nuclear shape (4.26 a). Cellular structure reveals that there is still sinusoidal dilation and mild necrosis of cells and less inflammation but cells are at recovering stage showing appearance of hepatocytes (figure 4.26 b). Figure 4.27(a, b) shows GWEF with treatment of 200 mg/kg and 400 mg/kg effects, exhibiting the mild fatty degeneration and protective effect with incation of intact central vein and well defined hepatic cells (hepatocyte) clearly separated by sinusoidal spaces (4.27a) while 4.27 (b) shows that there is more improvement of central vein appearance and well form of kupffer cells as well as epatocyte more clearly separated by sinusoidal spaces. This shows that GWEF has healing effects on liver cells. Figure 4.28 (a, b) represents the effects of 200 mg/kg and 400 mg/kg doses of GWBF after CCl_4 intoxication. A moderate cellular appearance having mild fatty degeneration and regeneration ability of hepatocytes with surrounding sinusoidal spaces are seen there (figure 4.28 a). There is also much improvement of liver cells showing the well-defined sinusoidal space and structure of cells. There is normal central vein showing that GWBF also has dose dependent protective effect against CCl_4 induced toxicity. Figure 4.29 (a, b) shows the result of GWAF treatments with 200 mg/kg and 400 mg/kg doses by CCl_4

intoxication respectively. Figure 4.29 (a) shows clear central vein, clear hepatocytes having nuclei and well separated by sinusoidal space. Figure 4.29 (b) shows that there is much protective effect of GWAF on dose dependent manner characterized by very clear central vein (CV) and normal cellular architecture with very clear separation by sinusoidal spaces. There are also kupffer cells showing that GWAF has shown highest protective effect following with GWEF and GWBF on dose dependent manners.

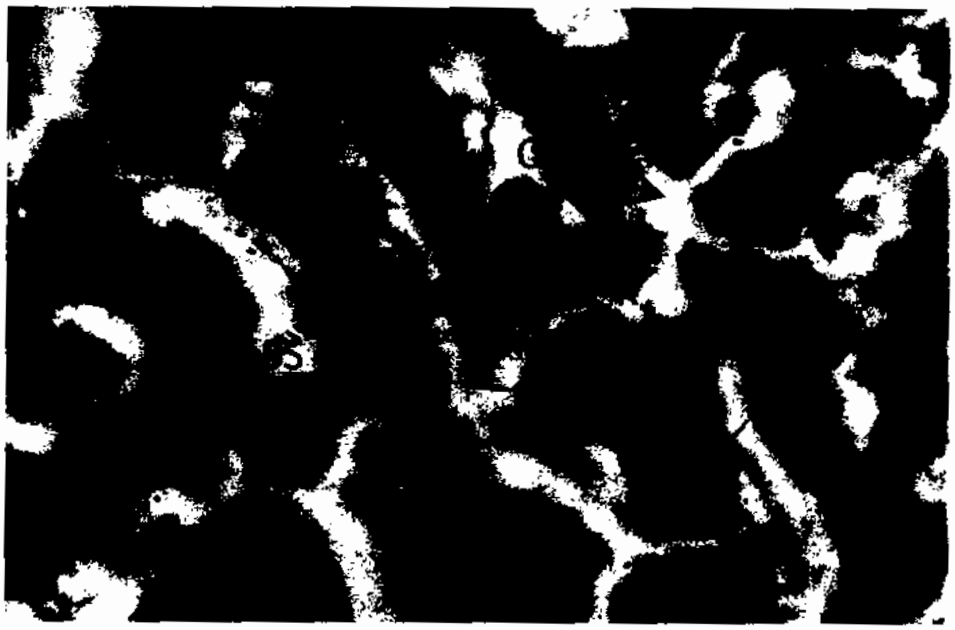


Figure 4.22: Photomicrograph of Liver Section of NC Mice (G-I)

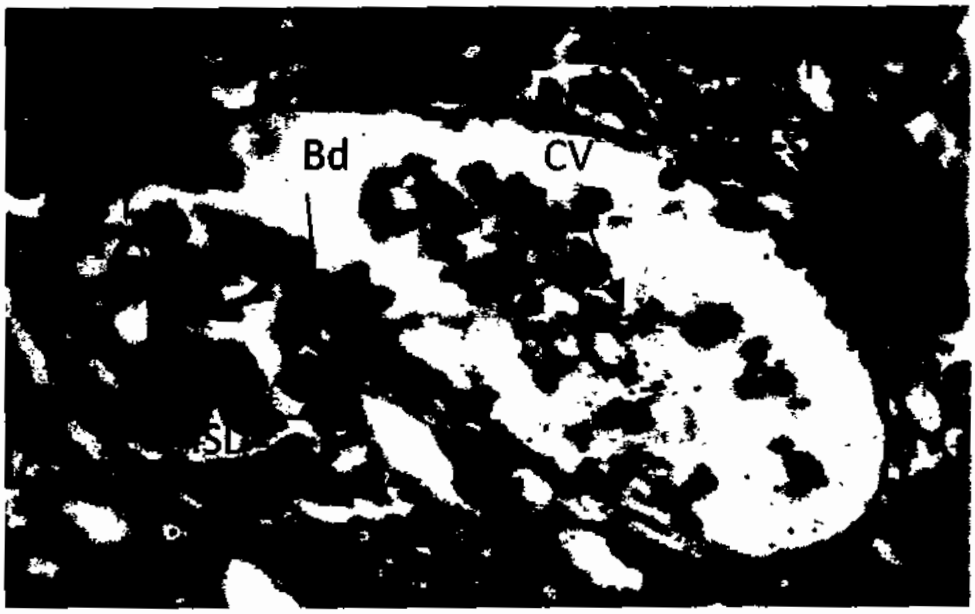


Figure 4.23: Photomicrograph of Liver Section of TC (25% CCl_4) Mice (G-II)

Figure 4.24: Photomicrograph of Liver Section of G-III: CCl_4+ SD(100 mg/kg)

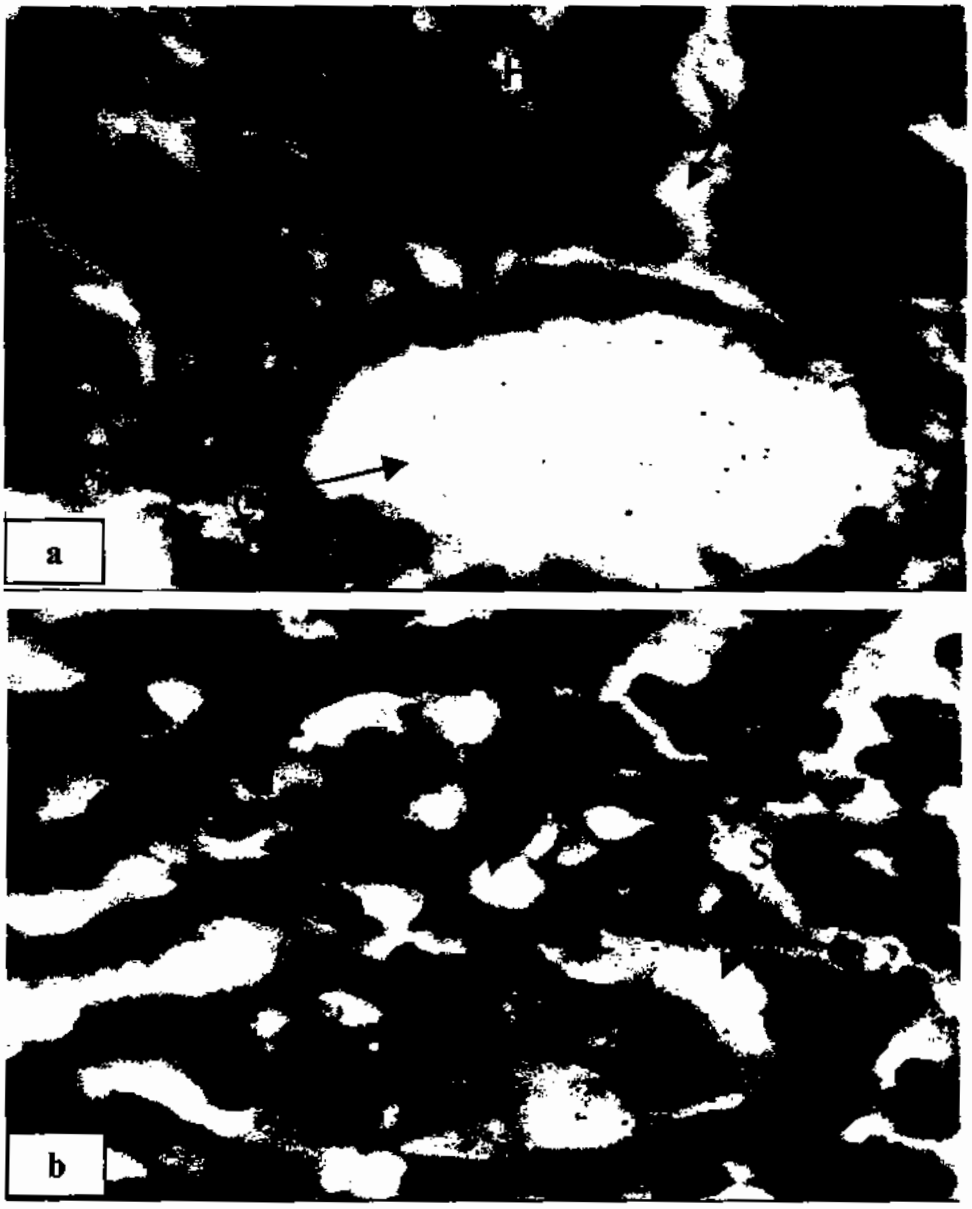


Figure 4.25: Photomicrograph of Liver Section of G-IVA: CCl_4+ GWHF 200mg/ kg (a) and G-VA: CCl_4+ GWHF (400mg/ kg)

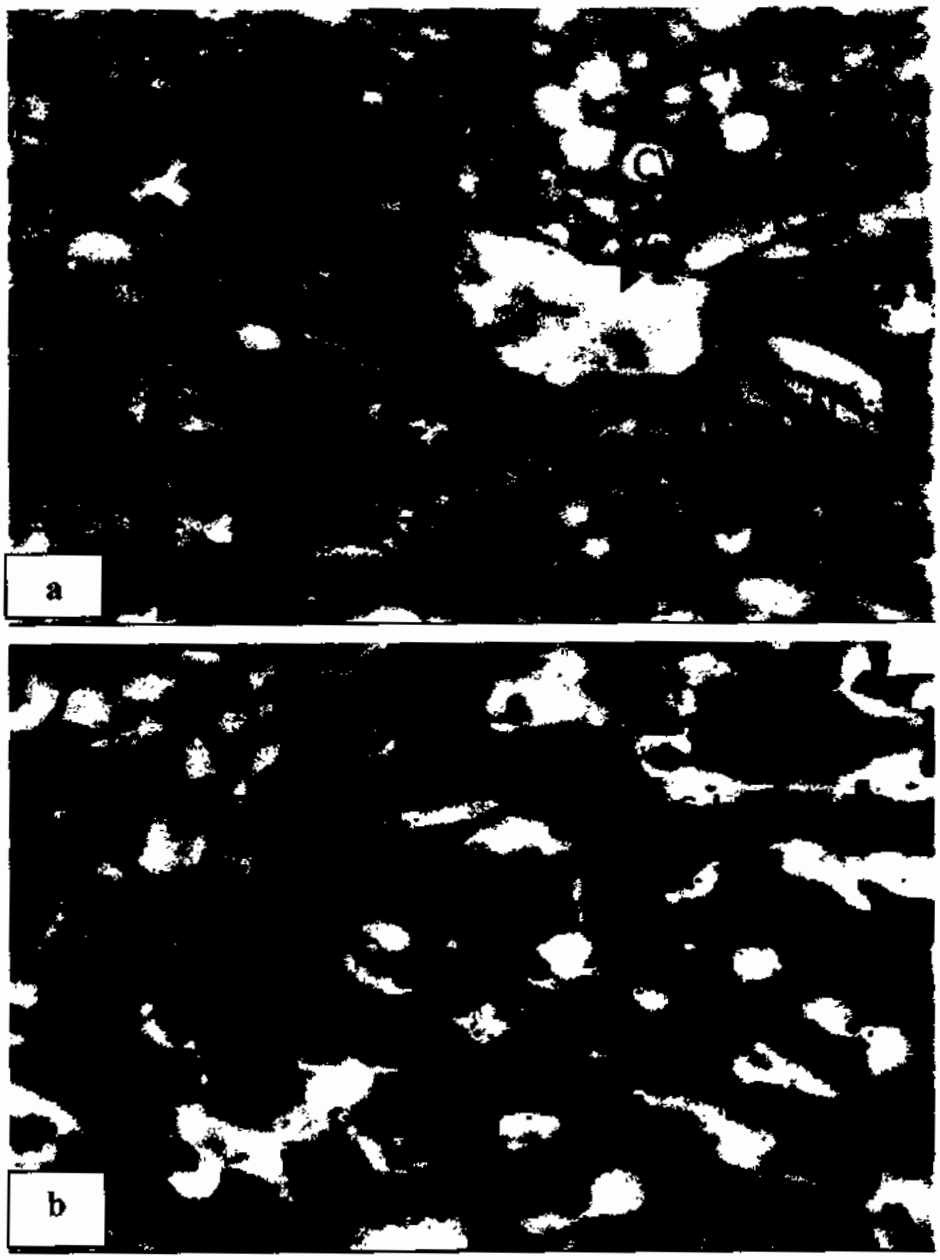


Figure 4.26: Photomicrograph of Liver Section of G-VIA: CCl_4+GWCF 200 mg/kg (a) and VIIA: CCl_4+GWCF 400 mg/kg (b)

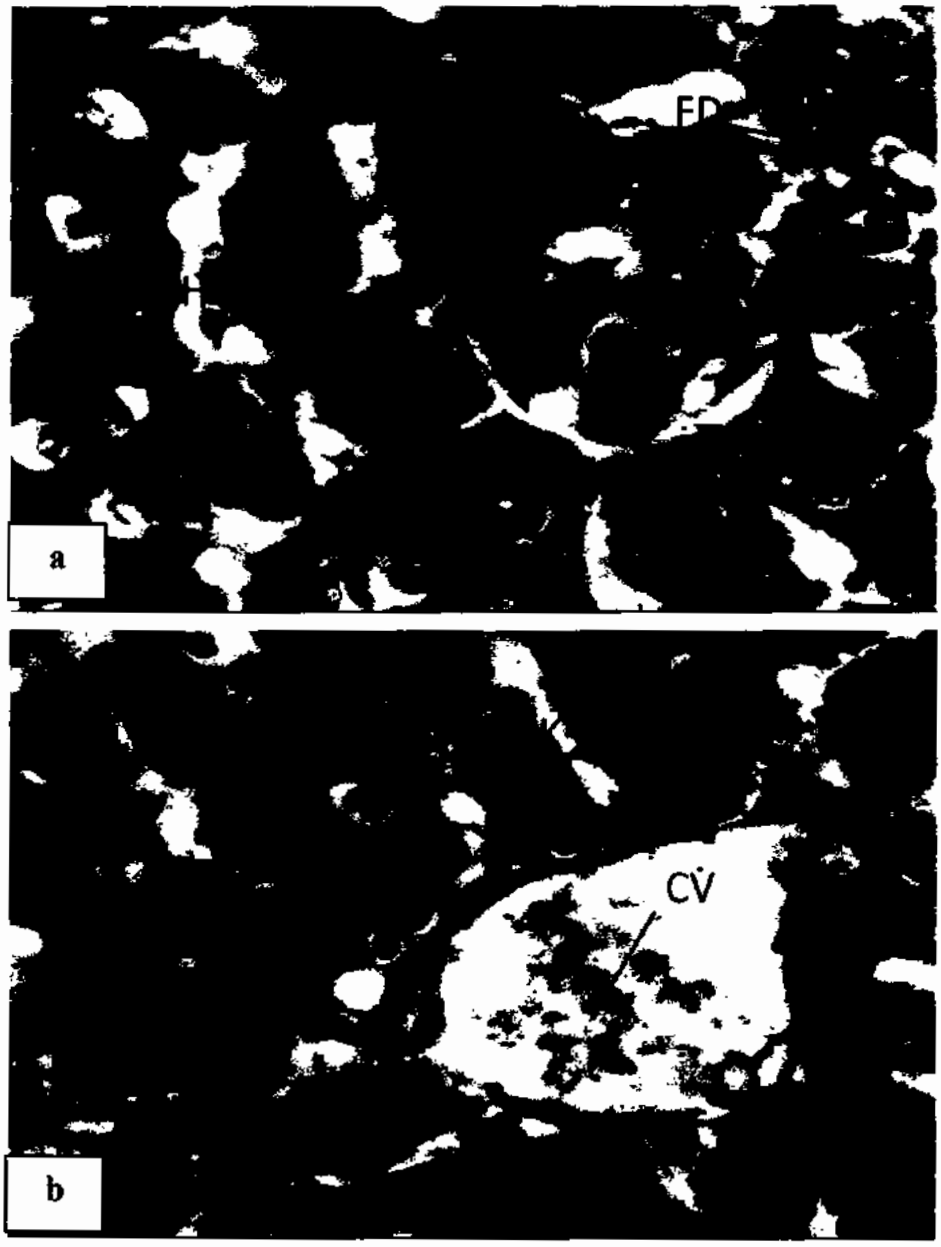


Figure 4.27: Photomicrograph of Liver Section of G-VIIIA: CCl_4+ GWEF 200 mg/kg (a) and G-IXA: CCl_4+ GWEF 400 mg/kg (b)

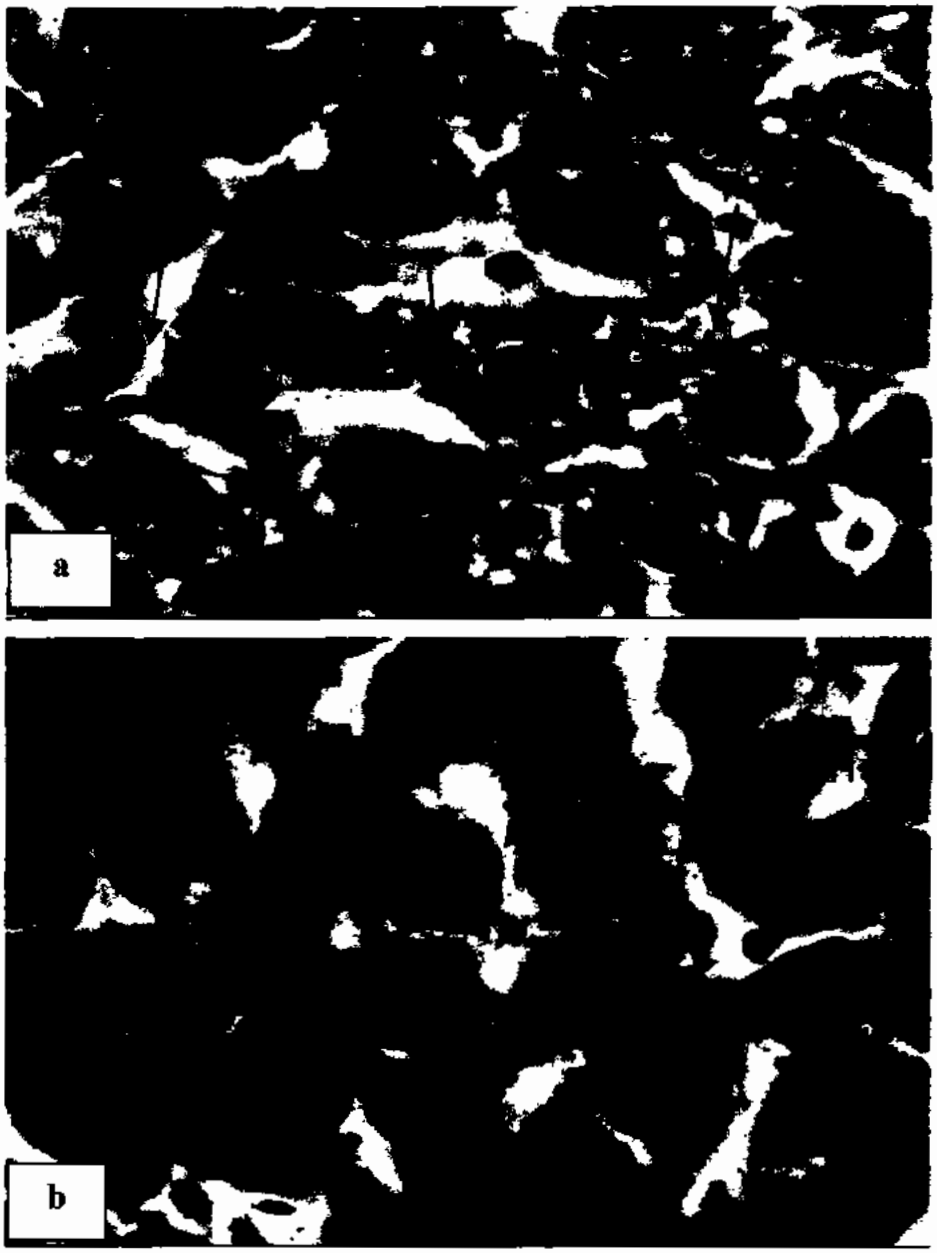


Figure 4.28: Photomicrograph of liver section of G-XA: CCl_4+GWBF 200 mg/kg (a) and G-XI: CCl_4+GWBF 400 mg/kg (b)

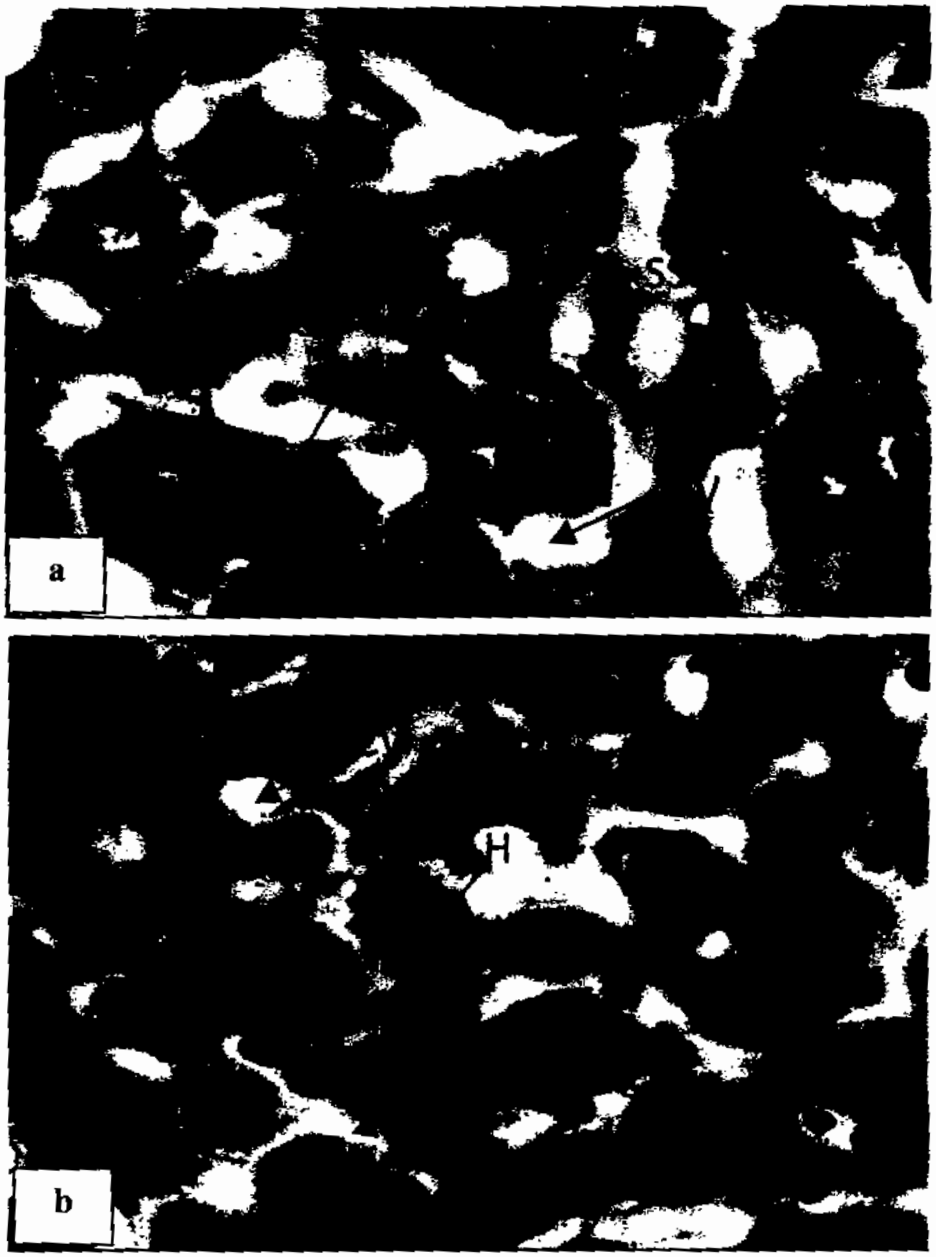


Figure 4.29: Photomicrograph of Liver Section of G-XIIA: CCl_4+GWAF 200 mg/kg (a) and XIIIA: CCl_4+GWAF 400 mg/kg (b)

H&E magnification100X (figure 4.22-4.29). Arrows show: CV- ventral vein; SS-sinusoids; H-hepatocytes; Bd-bleeding; FN- focal necrosis; I-inflammatory cellsinfiltration; P-portal vein; Sd-sinusoidal dilation and FD-fatty degeneration and K-kupffer cell.

4. 5. 2. Hepatoprotective Effects of Different Fractions of *E. parvifolia*

4. 5. 2.1. Liver Biochemicals Parameters

Results of *EP* fractions (EPHF, EPCF, EPEF, GWBF and EPAF) with treatments of 200 mg/kg and 400 mg /kg of each fraction on liver parameters (ALT, AST, ALP and TB) are shown by figures (4.30-4.33) below. Values of ALT by effects of 200 mg/kg of EP fractions (EPHF, EPCF, EPEF, EPBF and EPAF), orally administered in treatment groups (IVB, VIB, VIIIB, XB and XIIB) after CCl_4 intoxication significantly ($P < 0.01$) decreased to 171.12±4.08, 162.13±4.53, 121.03±5.20, 138.12±4.47 and 105.06±2.74 respectively as compare to T.C (CCl_4) group (G-II) which further decreased to 160.12±4.55, 152.17±4.76, 87±4.49 and 79.08±4.86 with significant effects ($P < 0.01$) due to high dose treatment (400 mg/kg) of EPHF, EPCF, EPEF, EPBF and EPAF, orally administered in treatment groups (VB, VIIB, IXB, XIB and XIIIB respectively) after CCl_4 intoxication respectively as compare to TC (CCl_4) group. It is evident that decrease effect is dose dependent with fractions EPEF, EPBF and EPAF and non-significant ($P > 0.01$) with fractions EPHF and EPCF when compared among themselves and all fractions have significant effect as compare to CCl_4 toxicant control groups (G-II) as indicated in figure 4.30. On treatments of EPHF, EPCF, EPEF, EPBF and EPAF with 200 mg/kg dose, orally administered in treatment groups (IVB, VIB, VIIIB, XB and XIIB) after CCl_4 intoxication, the values of AST significantly ($P< 0.01$) decreased to 184.10±4.11, 174±3.90. 136.03±3.90, 152.12±4.15 and 120.01±4.78 respectively as compare to TC (CCl_4) group. The values of AST further decreased to 170.10±5.98, 165.10±4.76, 104.04±4.34, 132.33±3.45 and 97.05±4.58 with significant ($P < 0.01$) effects by 400 mg/kg doses of EP fractions (EPHF, EPCF, EPEF, EPBF and EPAF), orally administered in treatment groups (VB, VIIB, IXB, XIB and XIIIB) respectively as compared to TC (CCl_4) group (G-II). There is highest effect of EPAF followed by EPEF and EPBF as compare to other fractions and non-significant difference is observed with 200 mg/kg and 400 mg/kg treatment of EPHF and EPCF when compared between themselves (figure 4.31). On treatments of 200 mg/kg doses of EPHF, EPCF, EPEF, EPBF and EPAF, orally administered in treatment groups (IVB, VIB, VIIIB, XB and XIIB respectively) after CCl_4 intoxication, the values of ALP significantly ($P< 0.01$) decreased to 363.50±4.36,

357.06±5.09, 288.02±4.40 and 291.09±4.61 respectively from TC (CCl_4) group (G-II). The values of ALP further decreased to 350.04±5.47, 344.09±5.54, 240.19±4.17, 265.11±4.75 and 220.04±4.06 with significant effects ($P < 0.01$) on 400 mg/kg doses of EPHF, EPCF, EPEF, EPBF and EPAF; orally administered in treatment groups (VB, VIIB, IXB, XIB and XIIIB) respectively as compare to TC (CCl_4) group (G-II). This shows the decreasing effect in dose dependent manner of EPAF followed by EPEF and EPBF and there is non-significant difference in EPHF and EPCF as compared between themselves and other fractions but significantly different as compare to TC (CCl_4) group (G-II) as indicated in figure 4.32. On treatments of 200 mg/kg dose of EPHF, EPCF, EPEF, EPBF and EPAF, orally administered in treatment groups (IVB, VIB, VIIIB, XB and XIIB) after CCl_4 intoxication, the values of TB significantly ($P < 0.01$) decreased to 1.98±0.16, 1.96±0.04, 142±0.04, 1.55±0.06 and 1.10±0.04 respectively as compare to CCl_4 toxicant control group (G-II). The values of TB further improved to decrease (1.85±0.12, 1.80±0.01, 1.03±0.06, 1.38±0.03 and 0.83±0.04) due to 400 mg/kg doses of EPHF, EPCF, EPEF, EPBF and EPAF; orally administered in treatment groups (VB, VIIB, IXB, XIB and XIIIB) respectively as compare to TC (CCl_4) group (G-II). It shows the decreasing effects in dose dependent manner as shown by the figure 4.33.

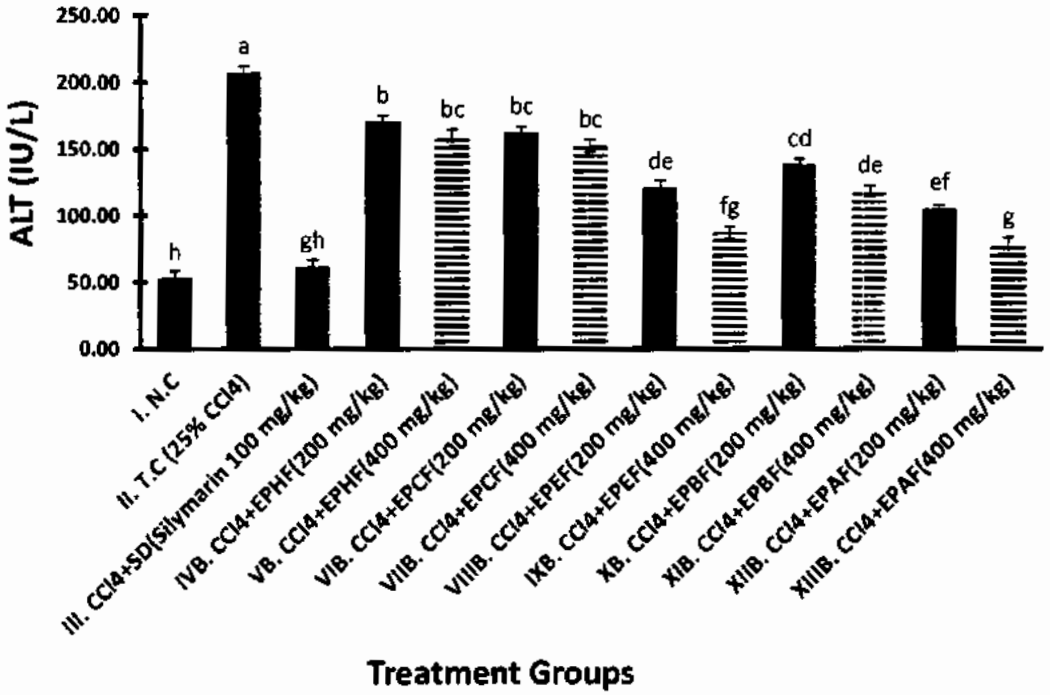


Figure 4.30: Effect of Different Fractions of *E. parvifolia* (EPHF, EPCF, EPEF, EPBF and EPAF) on ALT in Mice

Results are expressed as mean ± standard error of mean (n= 6). Mean values with different subscripts (a-h) letters are significantly ($P < 0.01$) different from each other while values with same letters are non-significantly ($P > 0.01$) different from each other.

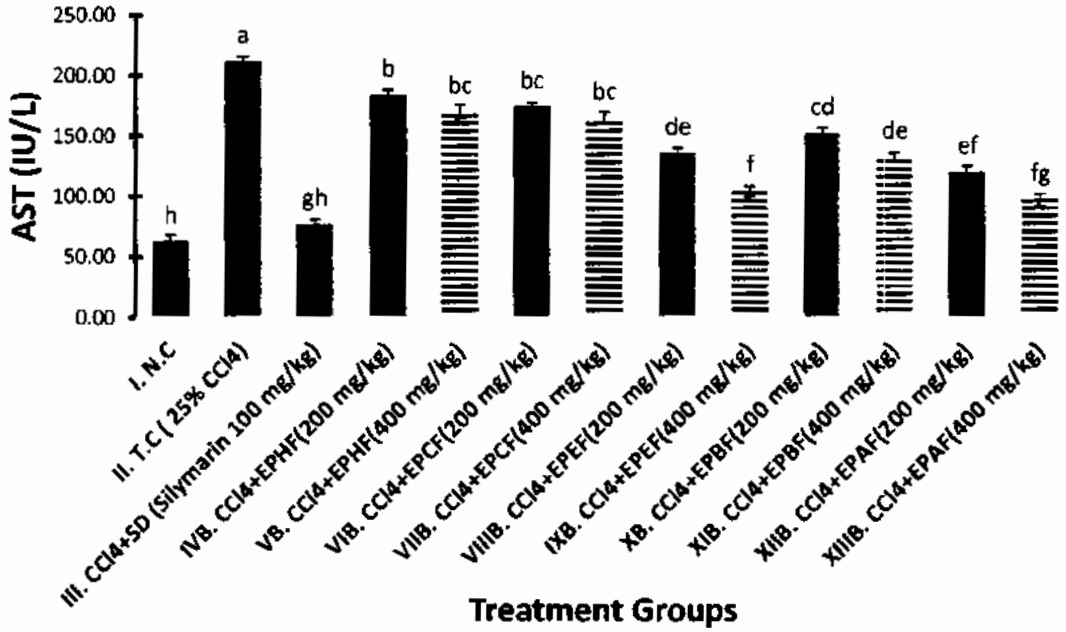


Figure 4.31: Effect of Different Fractions of *E. parvifolia* (EPHF, EPCF, EPEF, EPBF and EPAF) on AST in Mice

Results are expressed as mean ± standard error of mean (n= 6). Mean values with different subscripts (a-h) letters are significantly ($P < 0.01$) different from each other while values with same letters are non-significantly ($P > 0.01$) different from each other.

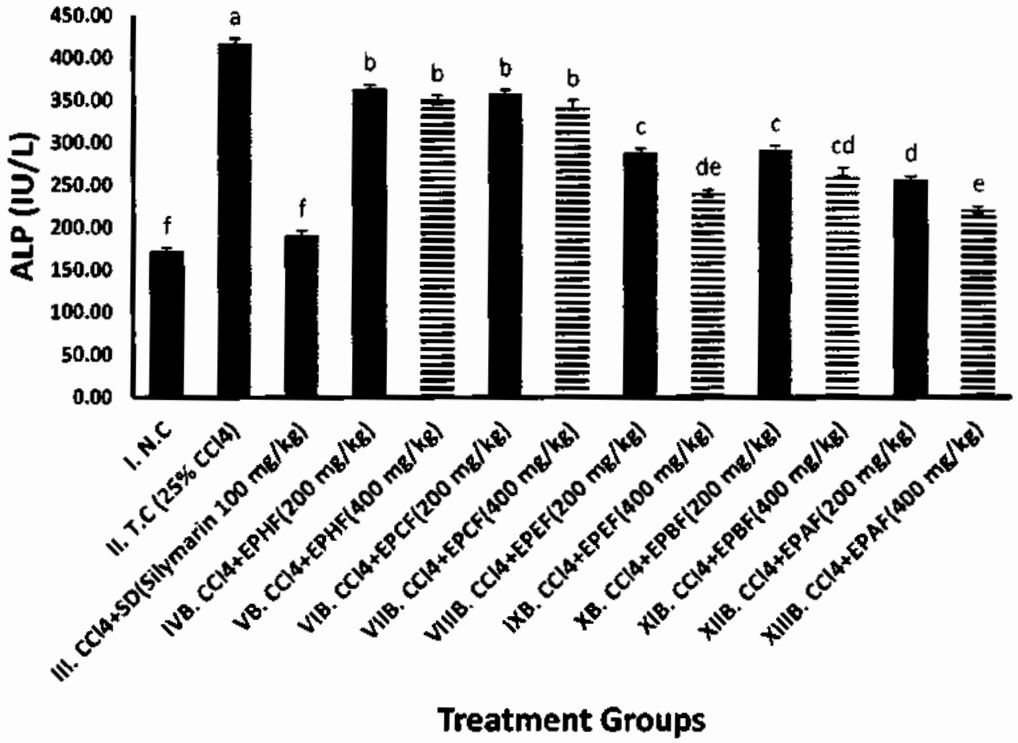


Figure 4.32: Effect of Different Fractions of *E. parvifolia* (EPHF, EPCF, EPEF, EPBF and EPAF) on ALP in Mice

Results are expressed as mean ± standard error of mean (n= 6). Mean values with different subscripts (a-f) letters are significantly ($P < 0.01$) different from each other while values with same letters are non-significantly ($P > 0.01$) different from each other.

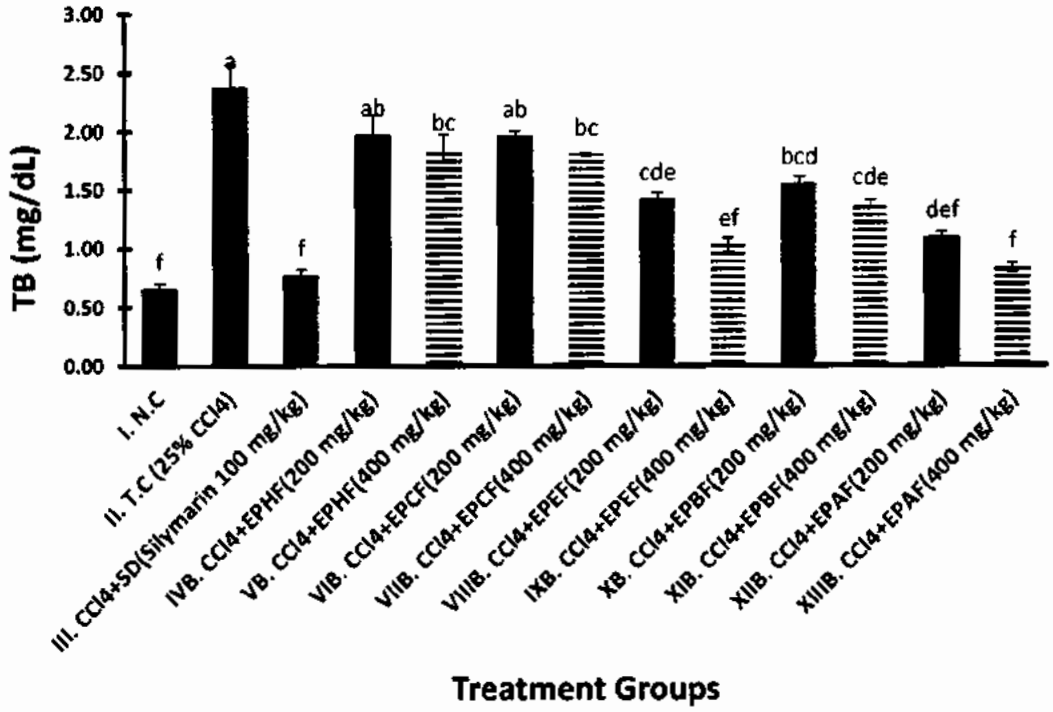


Figure 4.33: Effect of Different Fractions of *E. parvifolia* (EPHF, EPCF, EPEF, EPBF and EPAF) on TB in Mice

Results are expressed as mean ± standard error of mean (n= 6). Mean values with different subscripts (a-f) letters are significantly ($P < 0.01$) different from each other while values with same letters are non-significantly ($P > 0.01$) different from each other.

4.5.2.2 Liver Histopathology on Treatment of *EP* fractions

Microscopic investigations of liver histpathology of normal control mice, CCl_4 toxicant control (CCl_4) and SD (Silymarin 100 mg/kg) treatment are shown in figures 4.22-4.24. The results of histopathological changes due to effect of *E. parvifolia* fractions (EPHE, EPCF, EPEF, EPBF and EPAF) on liver sections in treatment groups (G-IVB- G-XIIIB) with 200 mg/kg and 400 mg/kg doses by CCl_4 induced toxicity are shown in figures 4.34-4.38 (a, b). Figure 4.34 (a, b) represents EPHF treatments effects with 200 mg/kg and 400 mg/kg against CCl_4 induced hepatotoxicity. Fatty degeneration and sinusoidal dilations are observed there. Hepatocytes are seen to recovering ability. There is mild necrosis and inflamatory cells infiltration.Central vein and hepatocytes are moderately recovered. The effects of EPCF (200 mg/kg and 400 mg/kg) on CCl_4 intoxication are shown by the figure 4.35 (a, b) showing fatty degeneration, ballooning, focal necrosis of hepatocytes and central cein in constricted shape but there is clear sinusoidal seperationof hepatocytes, showing that cells have recovering ability (4.35a). Cells structures exhibit that central vein is clearly present and well developed hepatocyte with clear seperation by sinusoids (4.35 b). Figure 4.36 (a, b) indicates the treatments effects of EPEF (200 mg/kg and 400 mg/kg doses) after CCl_4 intoxication revealing the mild fatty degeneration and also mild focal necrosis. Hepatocytes and central vein are more clear showing more protective effect (4.36 a). More improvement of central vein, presence of kupffer cells and intact hepatocytes, more clearly separated by sinusoids space like normal cells indicate that EPEF exhibits recovery effects on liver cells compareable to normal cells histopathological appearance (4.36b).

Figure 4.37 (a, b) indicates the effects of EPBF treatment (200 mg/kg and 400 mg/kg doses) against CCl_4 intoxication. There is moderate cellular appearance having less fatty degenerations and sinusoidal dilation with moderate cellular degeneration (4.37 a). Sinusoidal spaces, prominent cells structures and clear central vein incates the improvement effects of EPBF (4.37b). Figure 4.38 (a, b) shows the result of EPAF treatments (200 mg/kg and 400 mg/kg doses) effects against CCl_4 liver intoxication. Hepatocytes having prominent nuclei, distinctly separated by sinusoidal space and normal central vein are seen (4.38 a). Liver tissue shown in figure 4.38 (b) indicates very clear separation of hepatocyte by sinusoidal spaces and normal cells.

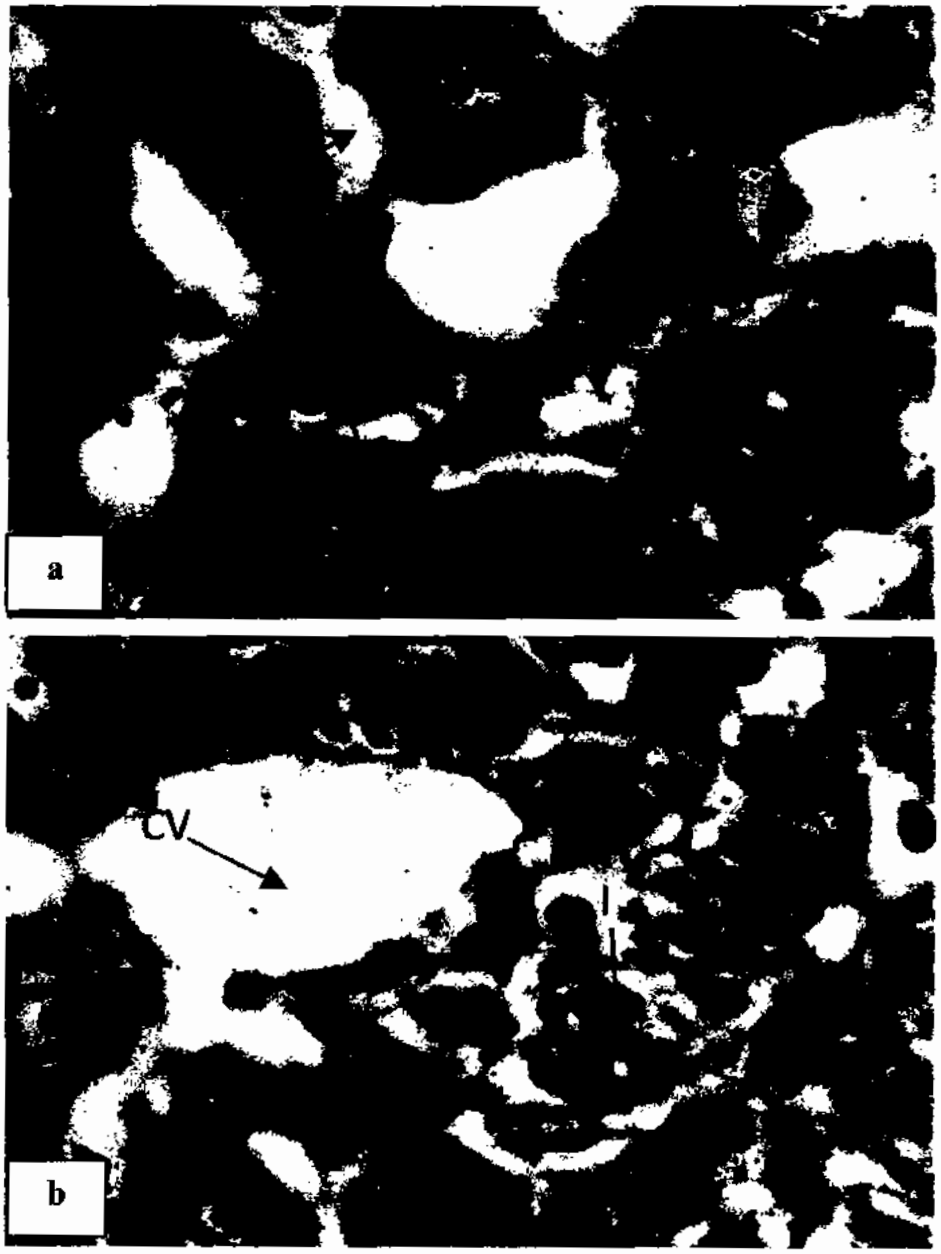


Figure 4.34: Photomicrograph of Liver Section of G-IVB: CCl_4+EPHF 200 mg/kg (a) and G-V-B: CCl_4+EPHF 400 mg/kg (b)

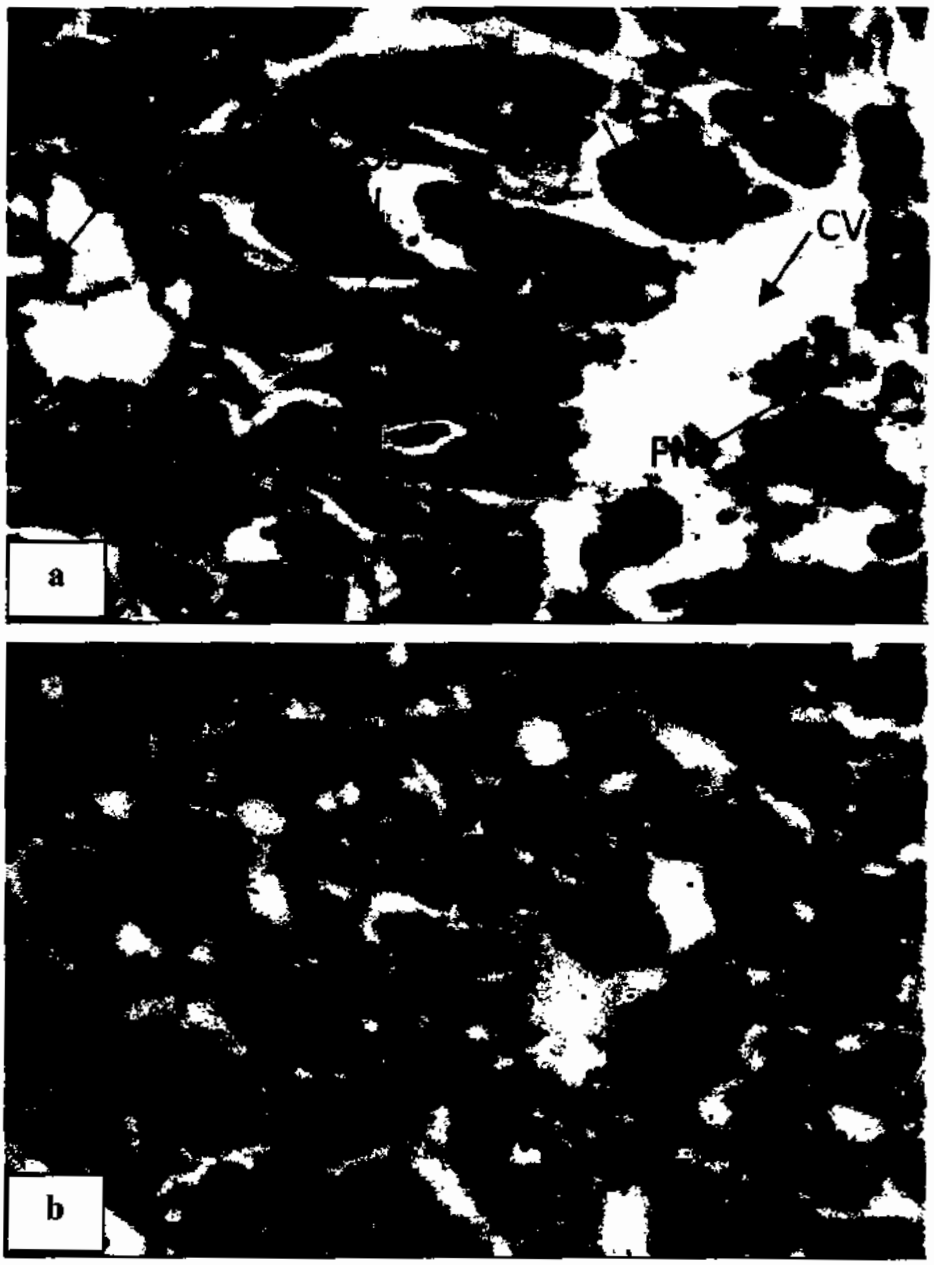


Figure 4.35: Photomicrograph of Liver Section of G-VIB: CCl_4+EPCF 200 mg/kg (a) and G-VIIB: CCl_4+EPCF 400 mg/kg (b)

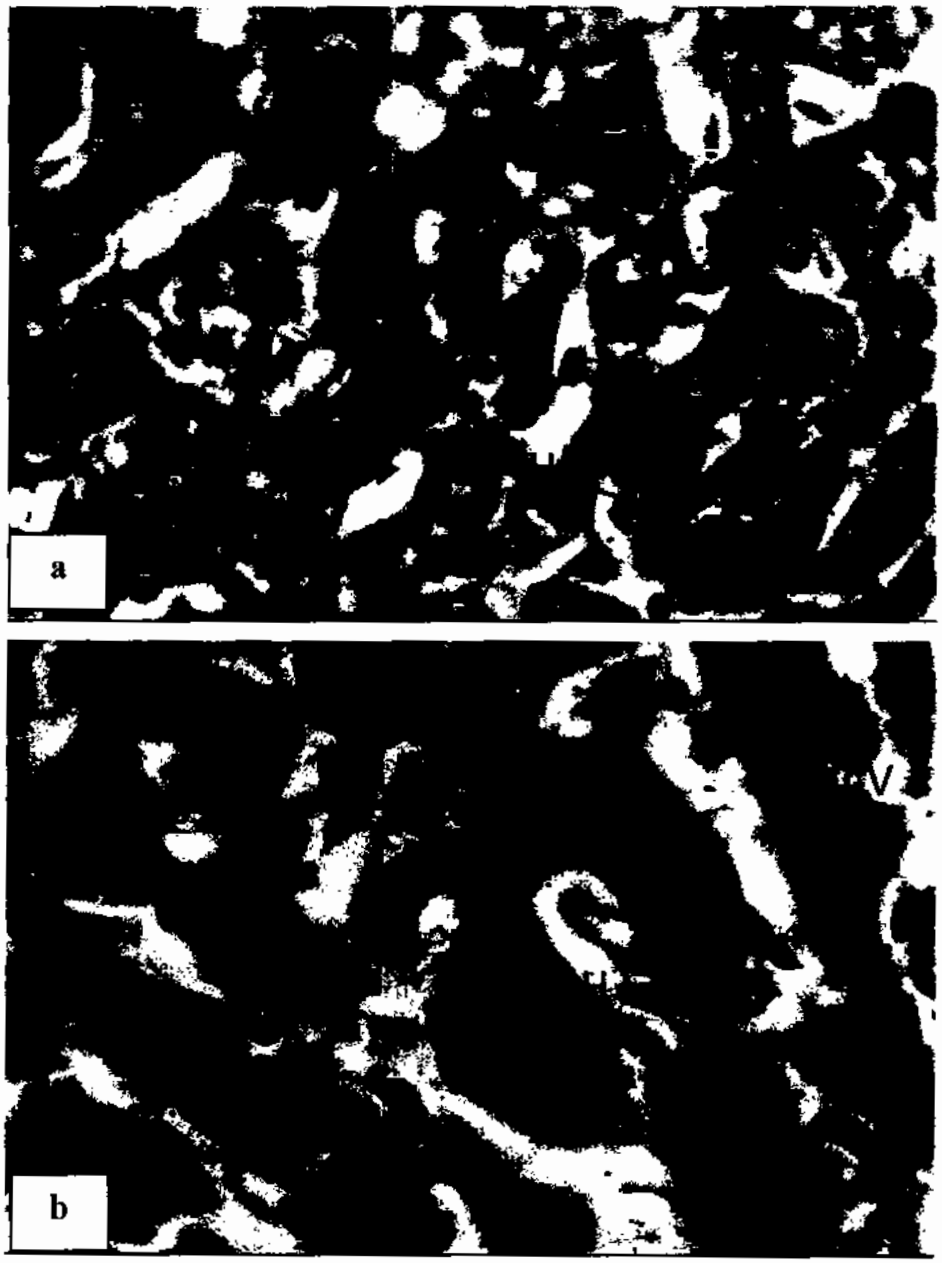


Figure 4.36: Photomicrograph of Liver Section of G-VIIIB: CCl_4+EPEF 200 mg/kg (a) and G-IX: CCl_4+EPEF 400 mg/kg (b)

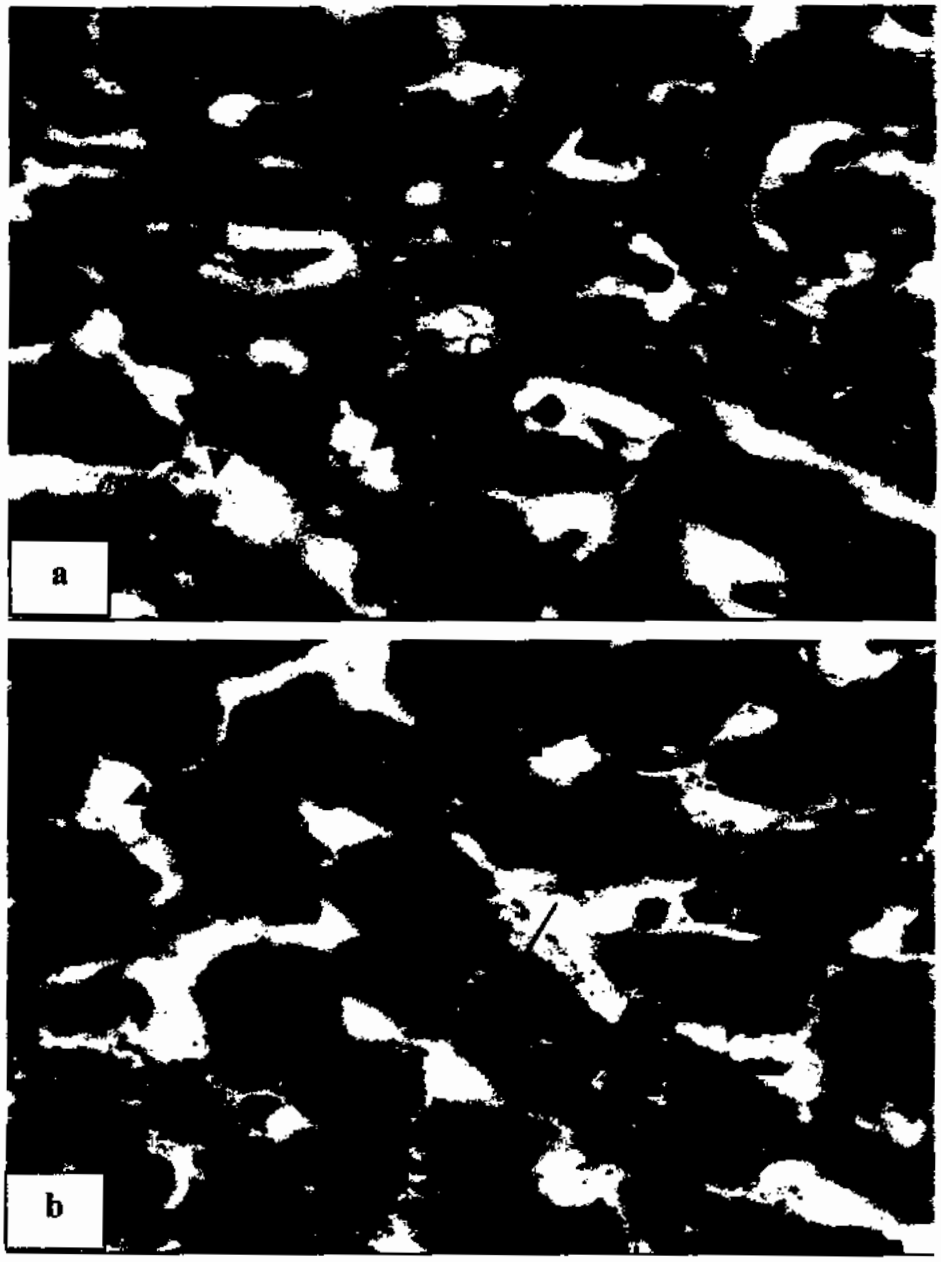


Figure 4.37: Photomicrograph of Liver Section of G-XB: CCl_4+EPBF200 mg/kg (a) and G-XI: CCl_4+EPBF400 mg/kg (b)

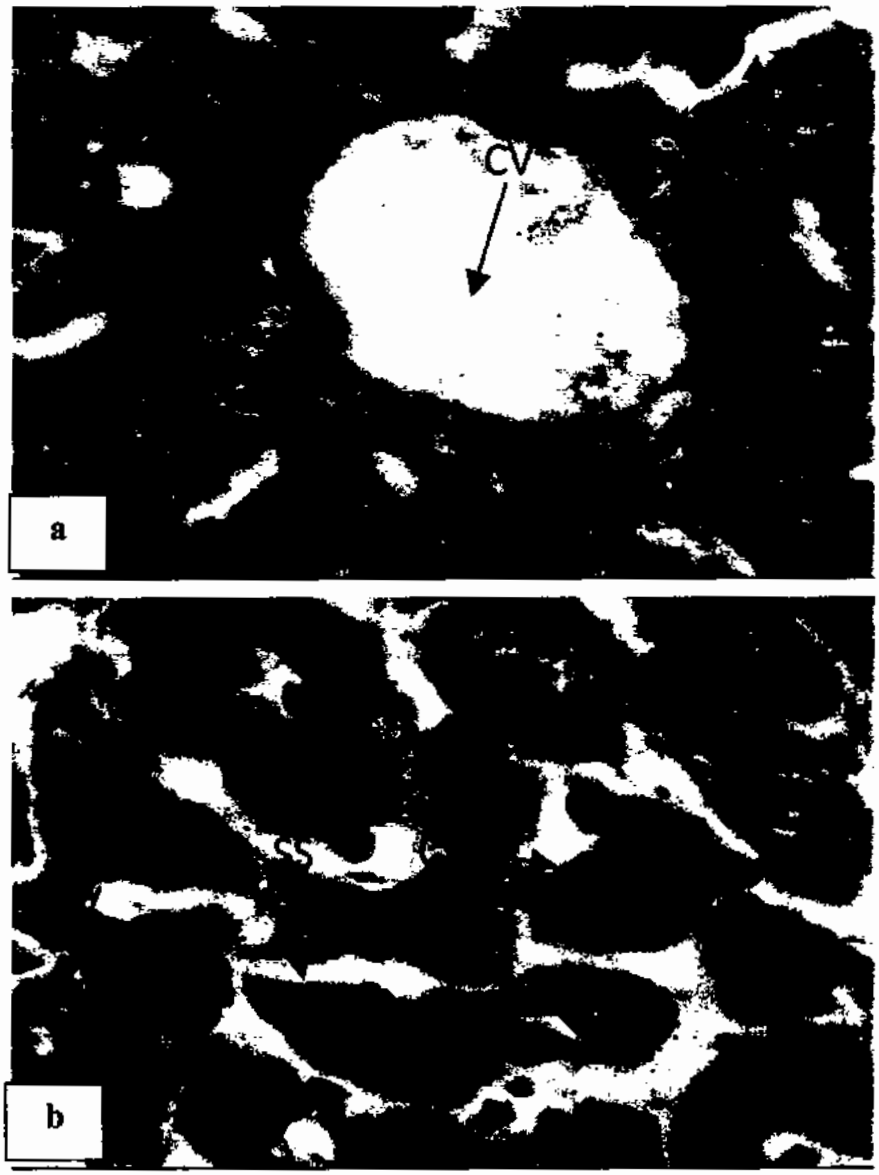


Figure 4.38: Photomicrograph of Liver Section of G-XIIB: CCl_4+EPAF200 mg/kg (a) and XIIIB: CCl_4+ EPAF400 mg/kg (b)

H&E magnification100X (figure 4.34-4.38). Arrows show: CV-ventral vein; SS- sinusoids; H-hepatocytes; Bd-bleeding; FN- focal necrosis; I-inflammatory cells infiltration; P-portal vein; Sd-sinusoidal dilation and FD-fatty degeneration and K-kupffer cell.

4.5.3 Hepatoprotective Effect of Different Fractions of *A. alpinum*

4.5.3.1 Liver Biochemical Parameters

Results of different fraction of *AA* (AAHF, AACF, AAEF, AABF and AAAF) by treatment of 200 mg/kg and 400 mg/kg) on ALT, AST, ALP (IU/L) and TB (mg/dL) against CCl_4 induced toxicity are shown by figures (4.39-4.42) below.The results show that 200 mg/kg of *AA* fractions (AAHF, AACF, AAEF, AABF and AAAF), with oral administration in treated groups (IVC, VIC, VIIIC, XC and XIIC), after CCl_4 intoxication; the values of ALT decreased (156.99±5.65, 160.06±5.85, 106.04±4.28,114.02±5.20 and 92.93±2.12 respectively) with sifnificant effects (P >0.01) as compare to TC (CCl_4) mice (G-II). ALT level further improved to decrease (144.15±5.58, 148.17±4.61, 73.03±5.19, 80.67±4.05 and 53.05±4.50 respectively) with treatment of 400 mg/kg doses of different fractions of *AA* (AAHF, AACF, AAEF, AABF and AAAF), oral administration in treatment groups (VC, VIIC, IXC, XIC and XIIIC) respectively as compare to CCl_4 toxicant control group (G-II). The dose dependent effect is non-significant (P > 0.01) with AAHF, AACF, and significant (P < 0.01) with treatment of AAEF, AABFandAAAF when compared among the fractions as indicated in figure 4.39. On oral administration of 200 mg/kg doses of different fractions of *AA* (AAHF, AACF, AAEF, AABF and AAAF) in treated groups (IVC, VIC, VIIIC, XC and XIIC) after CCl_4 intoxication; the values of AST significantly (P < 0.01) decreased (165.06±4.51, 175.05±3.75, 120.03±3.75, 128.17±2.90 and 107.05±3.46 respectively) as compare to T.C (CCl_4) mice (G-II). AST levels further decreased (160.01±5.32, 167.33±3.50, 90.04±5.05, 103.06±4.47 and 74.10±4.82 respectively) with treatment of 400 mg/kg dose of *AA* fractions (AAHF, AACF, AAEF, AABF and AAAF), in treatment groups (VC, VIIC, IXC, XIC and XIIIC) respectively as compare to CCl_4 toxicant control group (G-II). There is non-significant difference (P > 0.01) in AAHF and AACF when compared between each dose effect of same fraction and significant effect (P < 0.01) with AAEF, AABF and AAAF as compare to other fractions (figure 4.40).On oral administration of 200 mg/kg doses of different fractions of *A. alpinum* (AAHF, AACF, AAEF, AABF and AAAF), (IVC, VIC, VIIIC, XC and XIIC) after CCl_4 intoxication, the values of ALP significantly (P < 0.01)

decreased (358.05±5.24, 351.15±6.28, 250.11±4.27, 260.05±4.47 and 248.10±4.56), as compare to CCl_4 toxicant control group (G-II) respectively.

The value of ALP further decreased (345.11±5.22, 340.18±4.80, 194.03±3.46, 226.33±5.15 and 184.04±3.52 respectively) on effects of 400 mg/kg of *AA* fractions (AAHF, AACF, AAEF, AABF and AAAF), in treated grroups (VC, VIIC, IXC, XIC and XIIIC) respectively as compare to CCl_4 toxicant control group (G-II). It shows there is non-significant difference ($P > 0.01$) in treatment effect of AAHF and AACF and significant difference ($P < 0.01$) of AAEF, AABF and AAAF when compared between same fractions doses as indicated in figure 4.41.

The values of TB reduced (1.85±0.15, 1.80±0.03, 1.14±0.08, 1.22±0.06 and 1.09±0.03 respectively) with significant effects ($P < 0.01$) as compare to TC (CCl_4) mice (G-II) on oral administration of 200 mg/kg of *A. alpinum* fractions (AAHF, AACF, AAEF, AABF and AAAF), in treated groups (IVC, VIC, VIIIC, XC and XIIC) after CCl_4 intoxication. The value of TB further decreased (1.74±0.13, 1.68±0.02, 0.78±0.04, 0.92±0.05 and 0.58±0.05) on effects of 400 mg/kg of AAHF, AACF, AAEF, AABF and AAAF, in treated groups (VC, VIIC, IXC, XIC and XIIIC) respectively as compared to CCl_4 toxicant control group (G-II). AAHF and AACF has non-significant effect ($P > 0.01$) when compared between same fraction while AAEF, AABF and AAAF have significant ($P < 0.01$) effects on dose dependent manner as shown in figure 4.42.

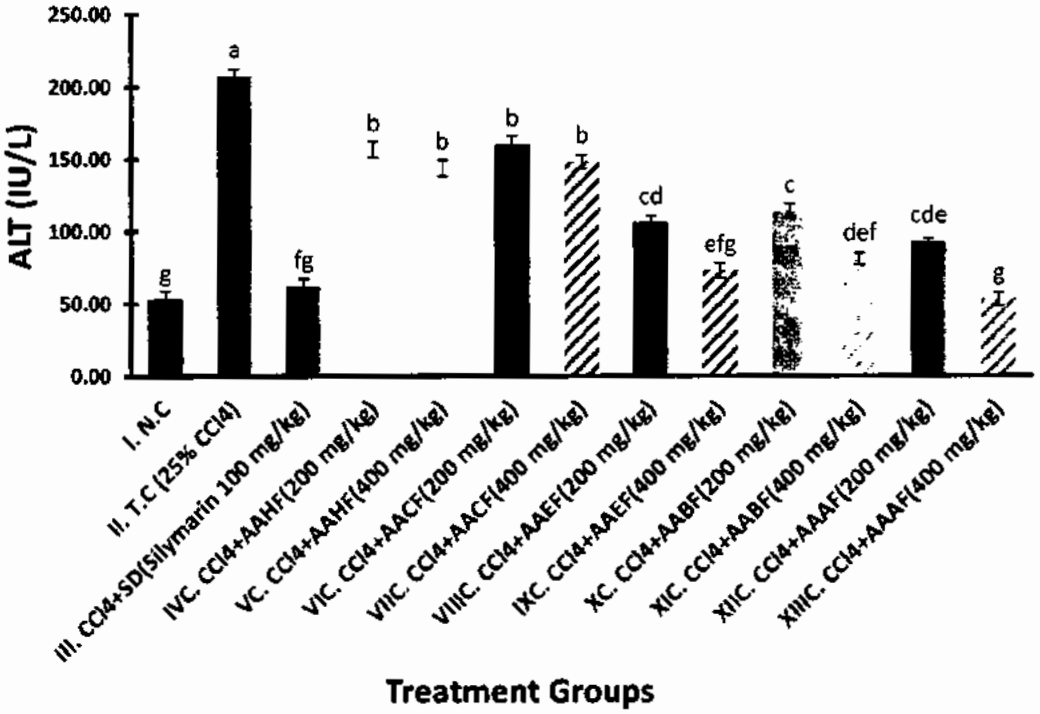


Figure 4.39: Effect of Different Fractions of *A. alpinum* (AAHF, AACF, AAEF, AABF and AAAF) on ALT in Mice

Results are expressed as mean ± standard error of mean (n= 6). Mean values with different subscripts (a-g) letters are significantly ($P < 0.01$) different from each other while values with same letters are non-significantly ($P > 0.01$) different from each other.

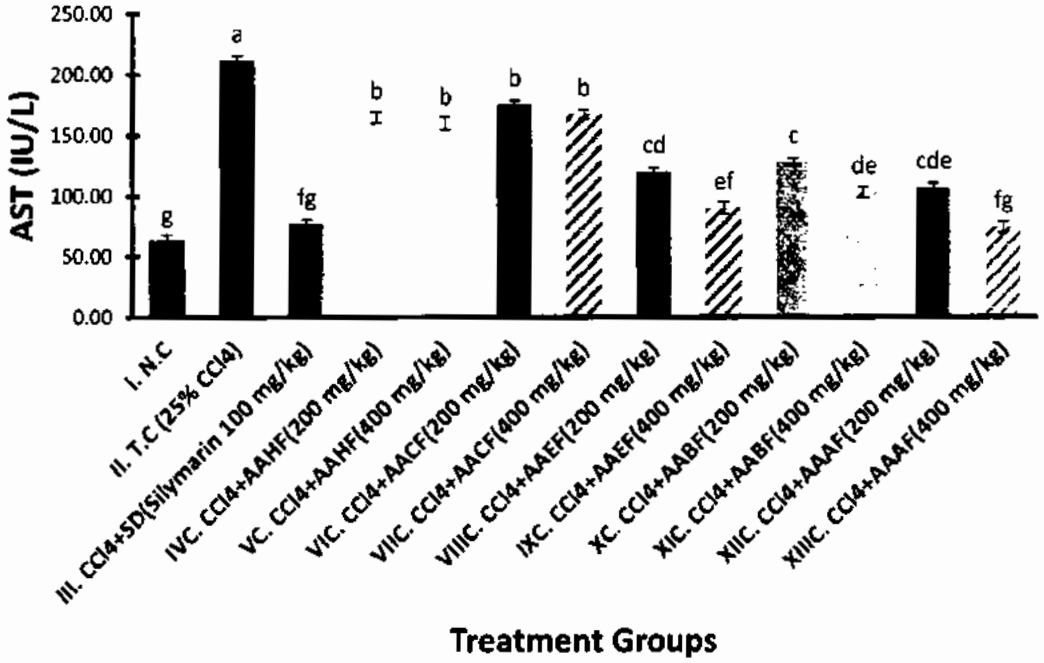


Figure 4.40: Effect of Different Fractions of A. alpinum (AAHF, AACF, AAEF, AABF and AAAF) on AST in Mice

Results are expressed as mean ± standard error of mean (n= 6). Mean values with different subscripts (a-g) letters are significantly ($P < 0.01$) different from each other while values with same letters are non-significantly ($P > 0.01$) different from each other.

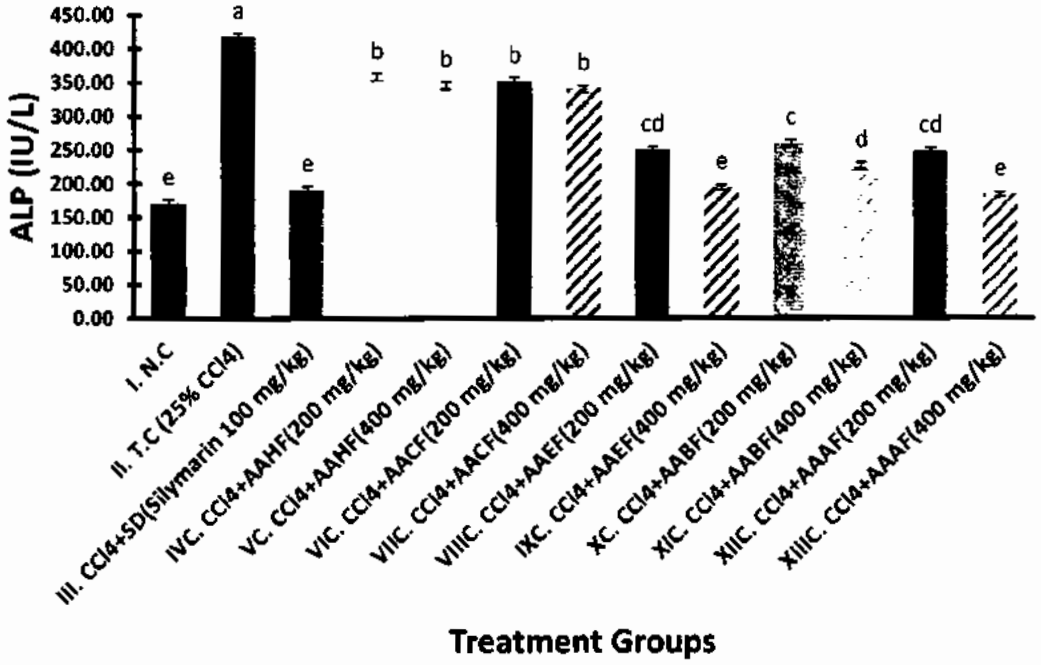


Figure 4.41: Effect of Different Fractions of *A. alpinum* (AAHF, AACF, AAEF, AABF and AAAF) on ALP in Mice

Results are expressed as mean ± standard error of mean (n= 6). Mean values with different subscripts (a-e) letters are significantly ($P < 0.01$) different from each other while values with same letters are non-significantly ($P > 0.01$) different from each other.

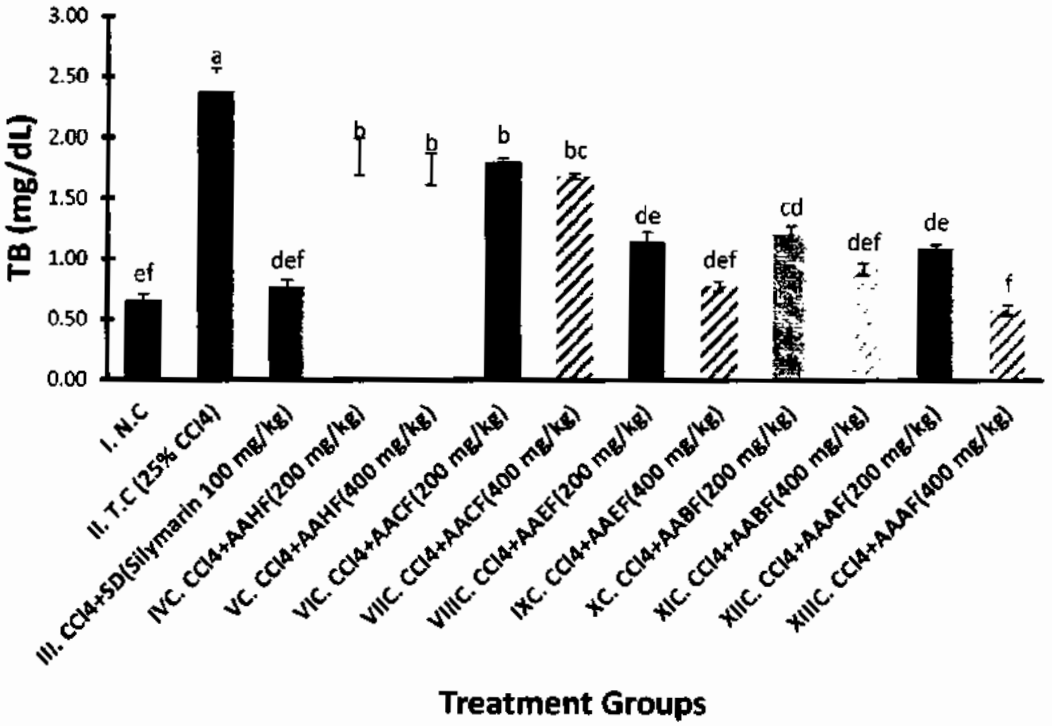


Figure 4.42: Effect of Different Fractions of *A. alpinum* (AAHF, AACF, AAEF, AABF and AAAF) on TB in Mice

Results are expressed as mean ± standard error of mean (n= 6). Mean values with different subscripts (a-f) letters are significantly ($P < 0.01$) different from each other while values with same letters are non-significantly ($P > 0.01$) different from each other.

4.5.3.2. Liver Histopathology on Treatment of *AA* Fractions

The results of histopathological changes due to effect of different fractions of *AA* (AAHF, AACF, AAEF, AABF and AAAF) on liver section with 200 mg/kg and 400 mg/kg doses in tratment groups (G-IVC- G-XIIIC), against CCl_4 induced toxicity in mice are shown in figures 4.43-4.47 (a, b). Figure 4.43 (a, b) shows AAHF 200 mg/kg and 400 mg/kg treatment effects. There is inflamatory cells infiltrations, necrosis, central vein constrictions

with less recovery of damaged cells (4.43 a). Moderate infiltrated inflamatory cells and fatty degeneration with moderate development (4.43 b). AACF treatments with 200 mg/kg and 400 mg/kg after CCl_4 induced toxicity are indicated by figure 4.44 (a, b) which shows that portal vein has dilated, less fatty degenerations occurred but clear seperation of hepatocyte having minimum loss in boudries of cells (4.44 a). The central vein and hepatocyte are going in progress of regeneration (4.44 b). Figure 4.45 (a, b) represents effects of AAEF with 200 mg/kg and 400 mg/kg doses treatments against CCl_4 toxicity; revealing distinctiction of hepatocyte and central vein with sinusoids space (4.45 a). More protective effect is exhibited by tissues having well appearance of kupffer cells, central vein, hepatocyte in normal condition and didtincted sinusoids spaces in organized form which is compareable to normal strcture of liver (4.45 b). Figure 4.46 (a, b) shows the effect of AABF with 200 mg/kg and 400 mg/kg doses against CCl_4 hepatotoxicity. Sinusoids and hepatocyte are also clearly distincted (4.46a). The damaged cells (hepatocyte) due to CCl_4 are in recovery stages and sinusoids have well formed spaces in liver cells (4.46b). The result of AAAF effects with 200 mg/kg and 400 mg/kg doses against CCl_4 are indicated in figure 4.47 (a, b). Central vein, hepatocyte and sinusoidal space are seen with normal shapes (4.47a) which are more clearly shown in figure 4.47 (b), with seperation of hepatocyte by sinusoids space which is compareable to NC group (figure 4.22).

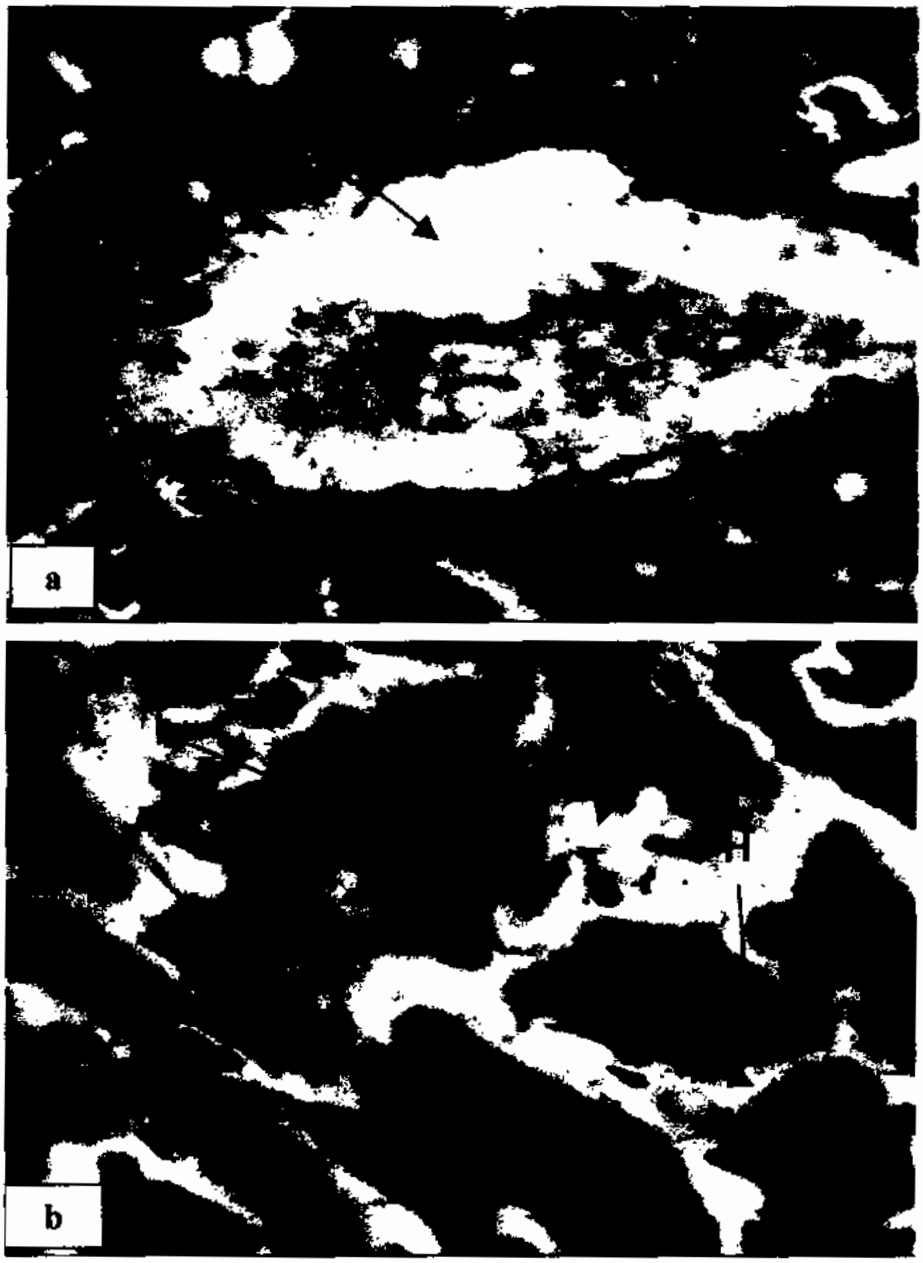


Figure 4.43: Photomicrograph of Liver Section of G-IVC: CCl_4+AAHF200 mg/kg (a) and G-VC: CCl_4+AAHF400 mg/kg (b)

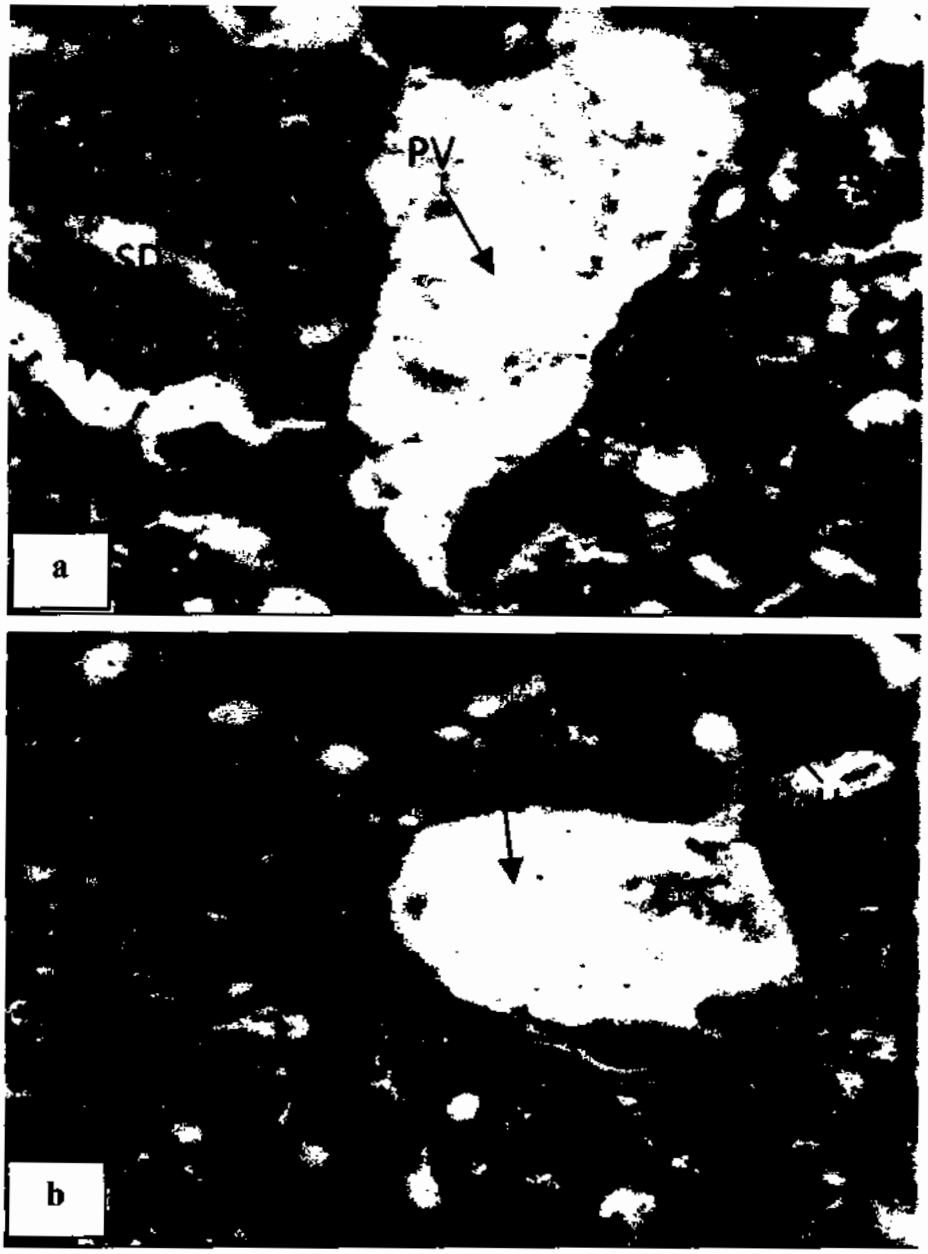


Figure 4.44: Photomicrograph of Liver Section of G-VIC: CCl_4+AACF 200 mg/kg (a) and G-VIIC: CCl_4+AACF 400mg/kg (b)

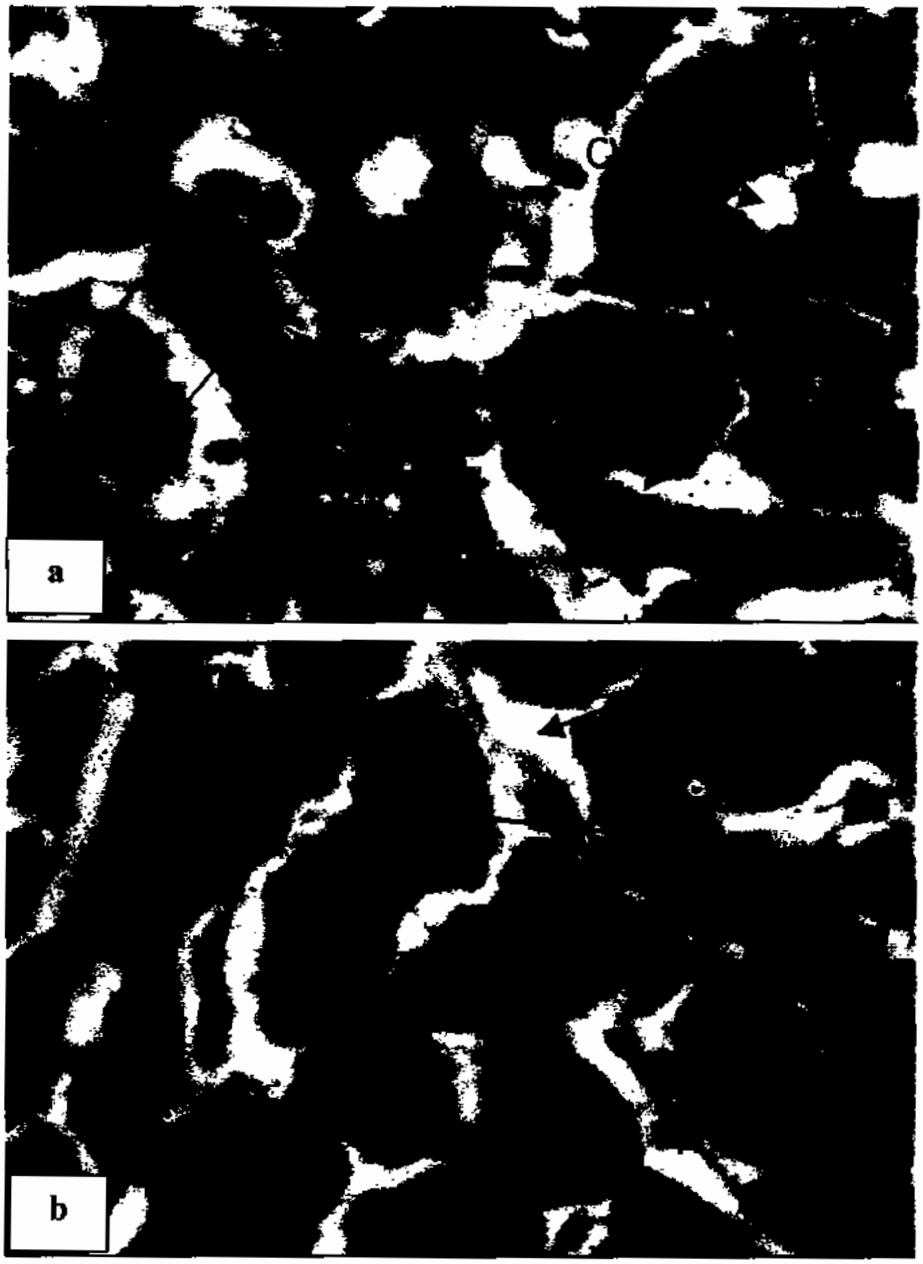


Figure 4.45: Photomicrograph of Liver Section of G-VIIIC: CCl_4+AAEF 200 mg/kg (a) and G-IXC: CCl_4+AAEF400 mg/kg (b)

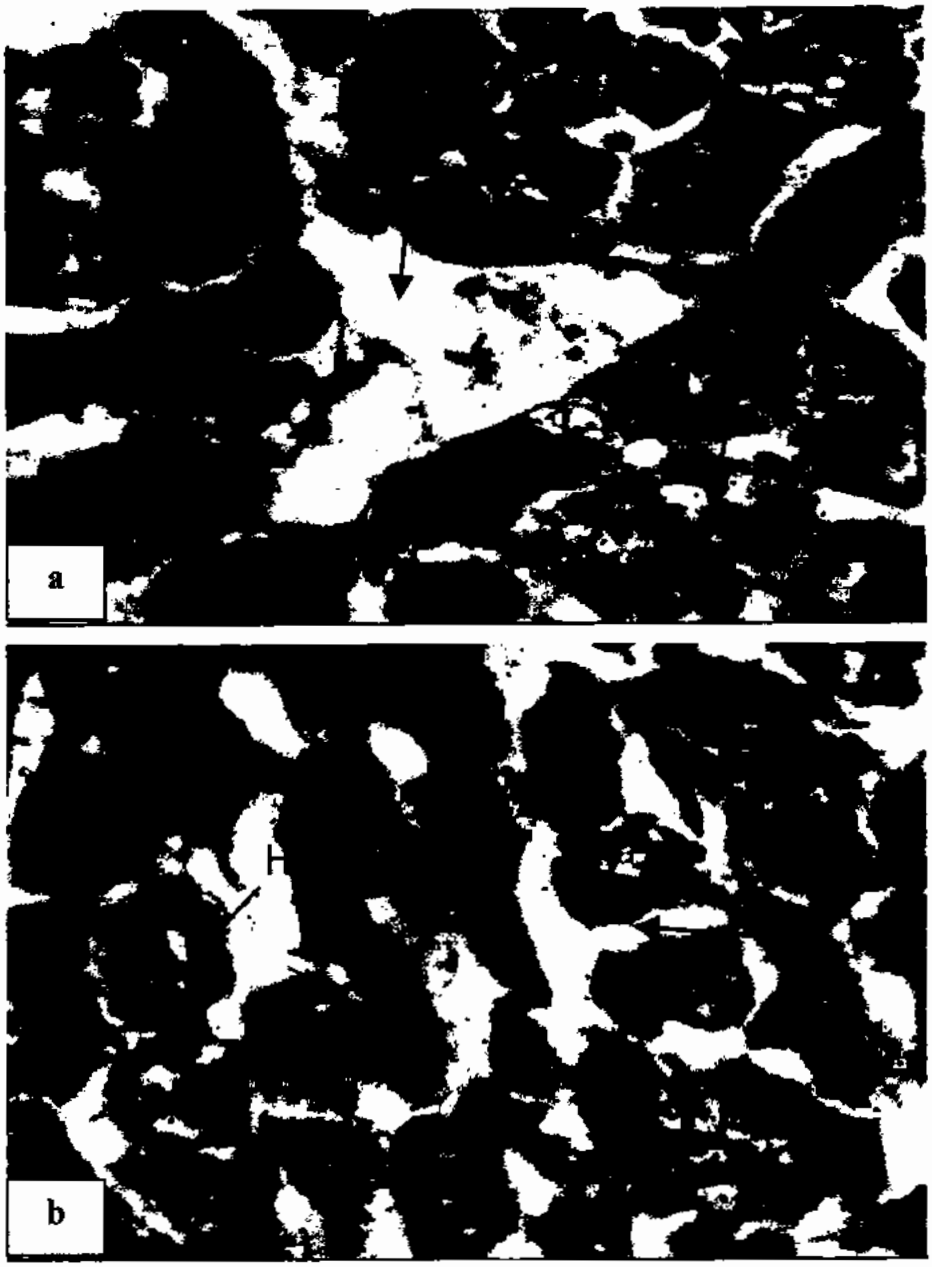


Figure 4.46: Photomicrograph of Liver Section of G-XC: CCl_4+AABF200 mg/kg (a) and G-XIC: CCl_4+ AABF 400 mg/kg (b)

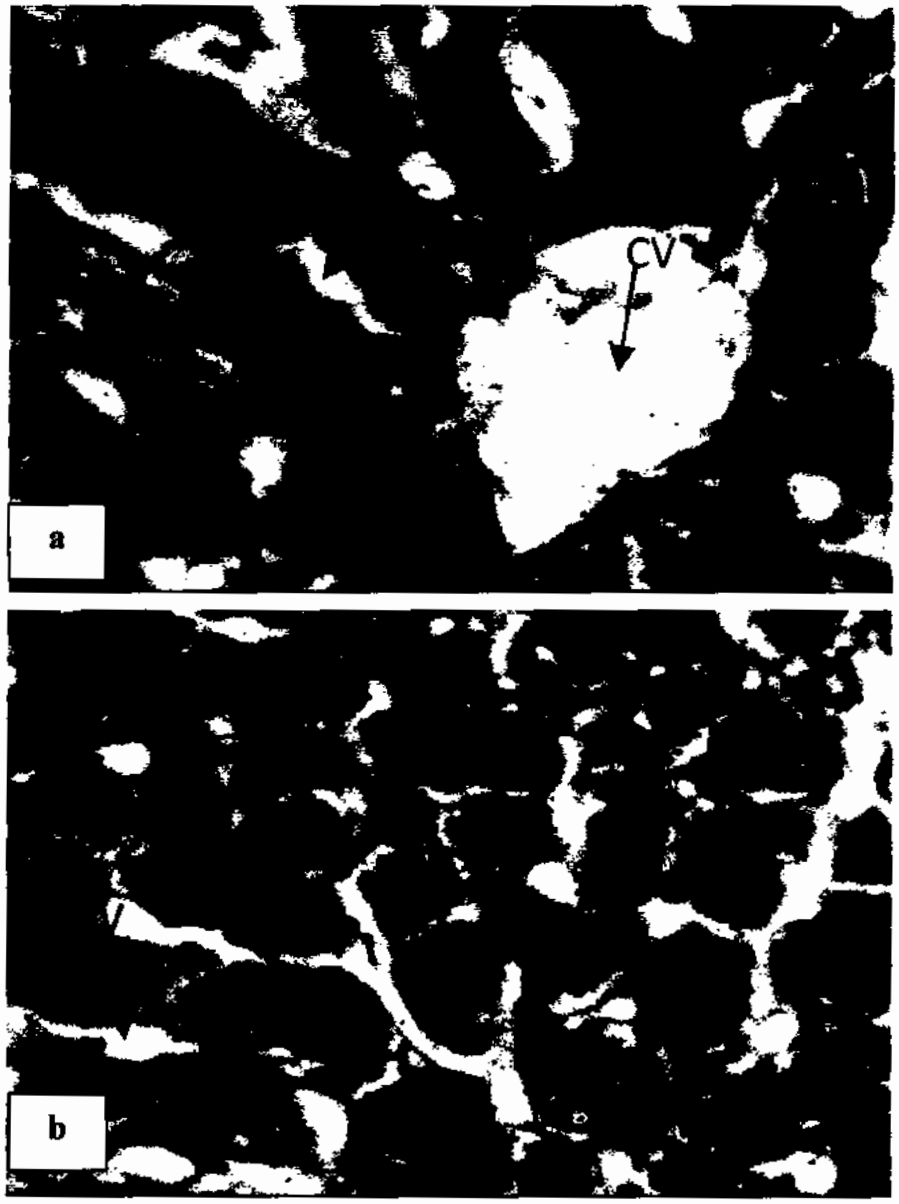


Figure 4.47: Photomicrograph of Liver Section of G-XIIC: CCl_4+AAAF 200 mg/kg (a) and G-XIIIC: CCl_4+AAAF 400 mg/kg (b)

H&E magnification100X (figure 4.43- 4.47). Arrows show: CV-ventral vein; SS-sinusoids; H-hepatocytes; Bd-bleeding; FN-focal necrosis; I-inflammatory cells infiltration; P-portal vein; Sd-sinusoidal dilation and FD-fatty degeneration and K-kupffer cell.

Comparision among different fractions themselves and with toxicant control group of three studied plants shows that there is non-significant difference ($P > 0.01$) in some fractions of *G. wallichianum* (GWHF and GWCF), *E. parvifolia* (EPHF and EPCF) and *A. alpinum* (AAHF and AACF) when compared between same fraction doses (200 mg/kg and 400 mg/kg), and significant difference when compared with CCl_4 toxicant control group as indicated in figures 4.18-4.21, 4.30-4.33 and 4.39-4.42 respectively.

4.5.4 Active Fractions of Selected Active Plants

The fractions of short listed active plants which exhibited effective role against CCl_4 intoxication in mice are indicated in the table 4.6 below. *G. wallichianum;* EF, BF and AF showed more hepatoprotective effect against CCl_4 intoxication in albino mice, in which EF and AF have highest activity. For *E. parvifolia*; EPEF and EPAF are most active against CCl_4 induced liver toxicity in albino mice. In case of *A. alpinum*; ethyl acetate, n-butanol and aqueous fractions showed highest hepatoprotective effect against CCl_4 induced liver toxicity in albino mice, in which ethylacetate, n-butanol and aqueous fractions are most active in *A. alpinum* extract.

Table 4.6: Active Fractions of Short Listed Active Plants

Plant	More Active Fractions	Most Active Fractions
G. wallichianum	GWEF	GWEF
	GWBF	
	GWAF	GWAF
E. parvifolia	EPEF	EPEF
	EPBF	
	EPAF	EPAF
A. alpinum	AAEF	AAEF
	AABF	AABF
	AAAF	AAAF

4.6. Antioxidant Activity of Methanlic Extract and Fractions of Short Listed Active Plant (DPPH Radical Scavenging Assay)

Scavenging activity of free radical (DPPH) for the analysis of of antioxidant activity of natural products from plants extracts and microbial sources is mainly applied. Determination in reduction of DPPH by gain of electron from antioxidant extract to hydrazine by conversion of unpaired electron to paired electrons is base of this assay. The absorbance of DPPH radical is decreased by antioxidant by donation of hydrogen. It is noticed that the deep purple color of DPPH radical is changed to yellow on reduction. DPPH assay of methanolic extract and different concentrations of short listed active plants; *G. wallichianum*, *E. parvifolia* and *A. alpinum* was estimated using ascorbic acid as standard. Absorbance was recorded for the selected concentrations (10 µg/mL, 15 µg/mL, 25 µg/mL, 50 µg/mL, 70 µg/mL and 100 µg/mL) of ascorbic acid. Percent inhibition (%) calibration curves was plotted for standard ascorbic acid (AA) as shown in figure 4.51. IC_{50} value (concentration of extract that inhibits the formation of DPPH radical by 50%) calculations were made from regression equation;

$$y = 0.7283X+26.76 \text{ and } R^2 = 0.9771.$$

The results of IC_{50} values of *G. wallichianum*, *E. parvifolia* and *A. alpinum* samples are represented in the tables (4.7- 4.9) below.

4.6.1. DPPH Radical Scavenging Activity of *G. wallichianum*

The scavenging effect (%) is increased with concentration of sample as shown in figure 4.48. Concentration dependent potential against DPPH exhibited by samples and standard (AA). GWME and fractions (GWHF, GWCF, GWEF, GWBF and GWAF) showed effective free radical scavenging effect (% inhibition). There is highest scavenging activity (%) of AA (95.3±1.2) followed by GWEF (92.3±0.6), GWBF (79.5±1.5), GWAF (74.2±1.4), GWME (72± 2.4), GWCF (70.5±1.5) and GWHF (60.2±0.3) as indicated in the figure 4.48 below. The IC_{50} calculation was performed by calibration curve with standard ascorbic acid concentrations (10µg/mL, 15µg/mL, 25µg/mL, 50µg/mL, 70µg/mL and 100µg/mL) as shown in table 4.7 below. In case of antioxidant effects of above samples; GWME, GWHF,

GWCF, GWEF, GWBF GWAF and AA have the IC_{50} value of 50.83 µg/mL,76.9 µg/mL, 70.53 µg/mL, 38.06 µg/mL 40.26 µg/mL and 29.92 µg/mL respectively. GWAF showed lowest IC_{50} value 29.92 µg/mL. Decreasing order of IC_{50} is; GWHF > GWCF > GWME > GWBF > GWEF > GWAF (having IC $_{50}$ values of 76.9 µg/mL 70.53 µg/mL, 50.83 µg/mL 40.26 µg/mL, 38.06 µg/mL and 29.92 µg/mL) respectively. The IC_{50} value of standard antioxidant ascorbic acid (AA) is 31.90 µg/mL. The lowest is IC_{50} value of sample; highest is its antioxidant activity.

Table 4.7: Antioxidant Activity (IC_{50} Value) of *G. Wallichianum* Extract/Fractions and Ascorbic Acid (AA)

Plant/Sample	Extract/Fractions	IC_{50}(µg/mL)
G. wallichianum	GWME	50.83
	GWHF	76.9
	GWCF	70.53
	GWEF	38.06
	GWBF	40.26
	GWAF	29.92
Standard Ascorbic Acid (AA)		**31.9**

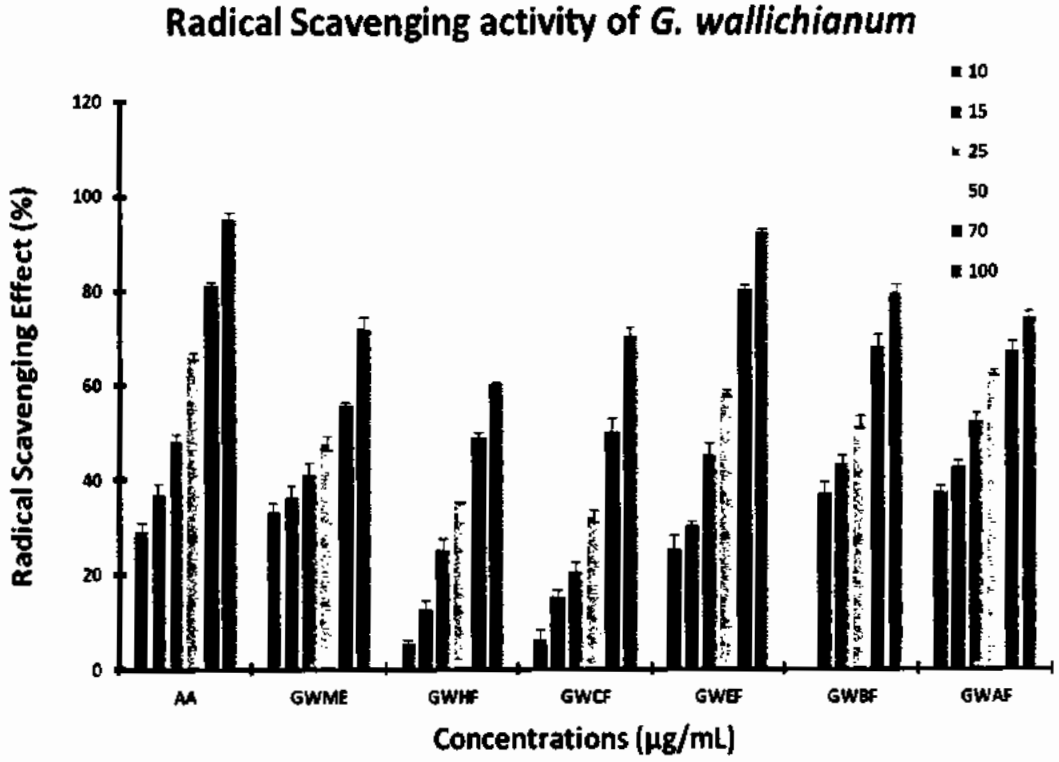


Figure 4.48: Concentration Versus Radical Scavenging Effect (%) of *G. wallichianum* Extract and Standard Ascorbic Acid (AA). Results are expressed as mean ± standard deviation (n=3).

4.6.2. DPPH Radical Scavenging Activity of *E. parvifolia*

Percent (%) scavenging effects of EPME and its different fraction are shown in figure 4.49. Highest scavenging activity is shown by AA (95.3± 1.2%), followed by EPEF (92.3±0.3), EPAF (82.5±1.5%), EPCF (75.2±1.3%), EPME (74.3± 2.3), EPBF (69.2 ±0.9 %) and EPHF (67.3±2.6 %) as shown in figure 4.49. IC_{50} calculation shows that in case of antioxidant activity of *EP*; EPME, EPHF, EPCF, EPEF, EPBF, EPAF and AA have the IC_{50} value of 51.01 µg/mL, 58.40 µg/mL, 57.84 µg/mL, 38.99 µg/mL, 53.95 µg/mL, 32.07 µg/mL and 31.90 µg/mL respectively as shown in table 4.8 below. The highest antioxidant activity

is shown by EPAF with lowest IC_{50} (32.07 µg/mL), which is comparable to IC_{50} value of ascorbic acid (31.90 µg/mL). Decreasing order of IC_{50} is; EPHF > EPCF > EPME > EPBF > EPEF > GWAF > AA (having IC_{50} values of 58.40 µg/mL, 57.84 µg/mL, 53.95 µg/mL, 51.01µg/mL, 38.99 µg/mL, 32.07 µg/mL and 31.90 µg/mL) respectively. Promising activity is of EPEF with IC_{50} value of 38.99 µg/mL. Then is activity of EPME and EPBF having IC_{50} value of 51.01 µg/mL and 53.95 µg/mL respectively. Less activity is exhibited by EPCF and EPHF having IC_{50} values 57.84 µg/mL and 58.40 µg/mL respectively as compare to AA standard activity (having IC_{50} value of 31.90 µg/mL).

Table 4.8: Antioxidant Activity (IC_{50} Value) of *E. parvifolia* Extract/Fractions and Ascorbic Acid (AA)

Plant/Sample	Extract/Fractions	IC_{50}(µg/mL)
E. parvifolia	EPME	51.01
	EPHF	58.40
	EPCF	57.84
	EPEF	38.99
	EPBF	53.95
	EPAF	32.07
Standard Ascorbic Acid (AA)		**31.9**

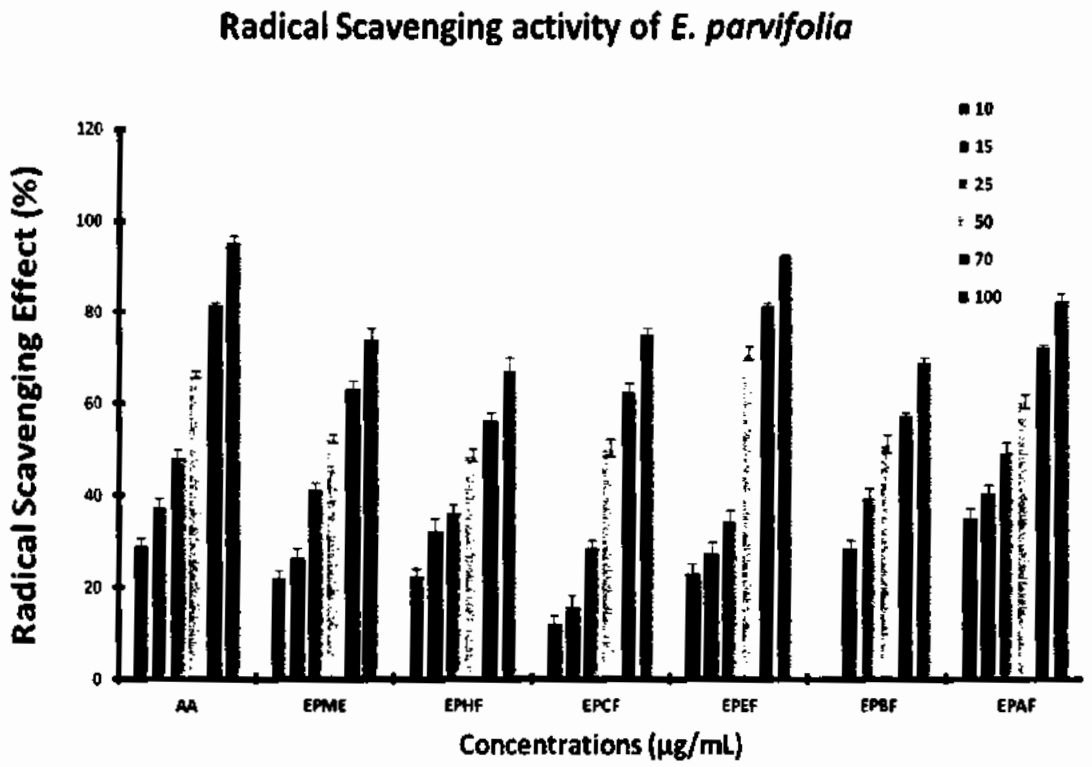


Figure 4.49: Concentration Versus Radical Scavenging Effect (%) of *E. parvifolia* Extract and Ascorbic Acid (AA). Results are expressed as mean+standard deviation (n=3).

4.6.3. DPPH Radical Scavenging Activity of *A. alpinum*

AAME and its different fractions (AAHF, AACF, AAEF, AABF and AAAF) exhibited effective scavenging activity to free radical. AAEF showed highest activity (96.4±0.6%) followed by AAME (91.2±1.4), AABF (89.5±0.2), AAAF (80.2±2.3), AACF (79.5±0.9) and AAHF (67. 2±2.4). The percent scavenging (%) effect was incrased on increasing concentrations of samples as shown by figure 4.50. For extracts samples (AAME, AAHF, AACF, AAEF, AABF, and AAAF) and standard ascorbic acid (AA), IC_{50} value are; 44.16 µg/mL, 73.75 µg/mL, 55.47µg/mL, 30.20µg/mL, 42.19 µg/mL, 25.51 µg/mL and

31.90 µg/mL respectively as shown in table 4.9 below. Decreasing order for IC_{50} value is; AAHF > AACF > AAME > AABF >AA> AAEF > AAAF (having IC_{50} values of 73.75 µg/mL, 55.47 µg/mL, 44.14 µg/mL, 42.19 µg/mL, 31.90 µg/mL, 30.20 µg/mL and 25.51 µg/mL) respectively. IC_{50} is inversely related to antioxidant activity (Low IC_{50} exhibits high antioxidant activity). AAAF showed lowest IC_{50} value (25.51 µg/mL) with highest antioxidant activity. Promising high antioxidant activity is of AAEF (IC_{50} 30.06 µg/mL) as compare to IC_{50} vale of ascorbic acid (31.90 µg/mL).

Table 4.9: Antioxidant Activity (IC_{50} value) of *A. alpinum* Extract/Fractions and Ascorbic Acid (AA)

Plant/Sample	Extract/Fractions	IC_{50}(µg/mL)
A. alpinum	AAME	44.16
	AAHF	73.75
	AACF	55.47
	AAEF	30.20
	AABF	42.19
	AAAF	25.51
Standard Ascorbic Acid (AA)		**31.9**

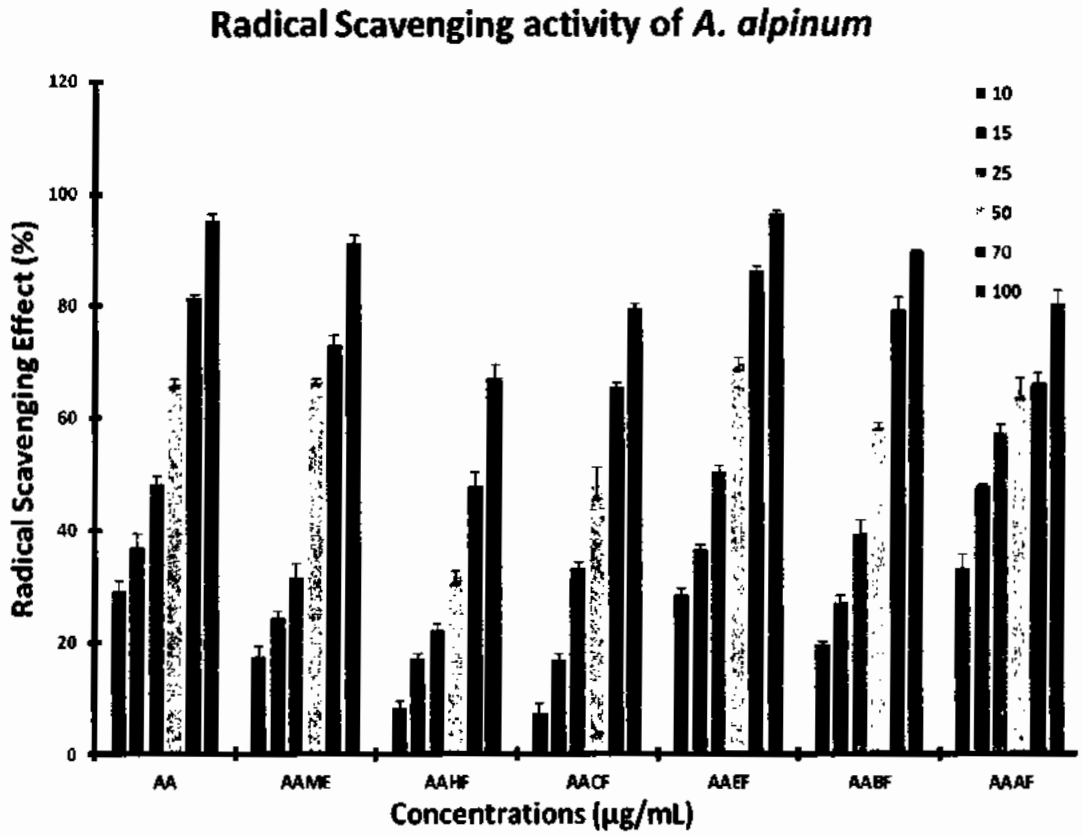


Figure 4.50: Concentration Versus Radical Scavenging Effect (%) of *A. alpinum* Extract and Standard Ascorbic Acid (AA). Results are expressed as mean+standard deviation (n=3).

The results of DPPH percent (%) scavenging activity of more active fractions of *G. wallichianum*, *E. parvifolia* and *A. alpinum* with IC_{50} values shown in tables (4.7-4.9) respectively can be summarized as; the highest antioxidant activity is of AAAF (IC_{50} value 25.51 µg/mL) followed by GWAF (29.92 µg/mL), AAEF (30.02 µg/mL), EPAF (32.07 µg/mL), GWEF (38.06 µg/mL), EPEF (38.99 µg/mL), GWBF (40.26 µg/mL) and AABF (42.19 µg/mL) as compare to antioxidant activity of AA with IC_{50} value 31.90 µg/mL.

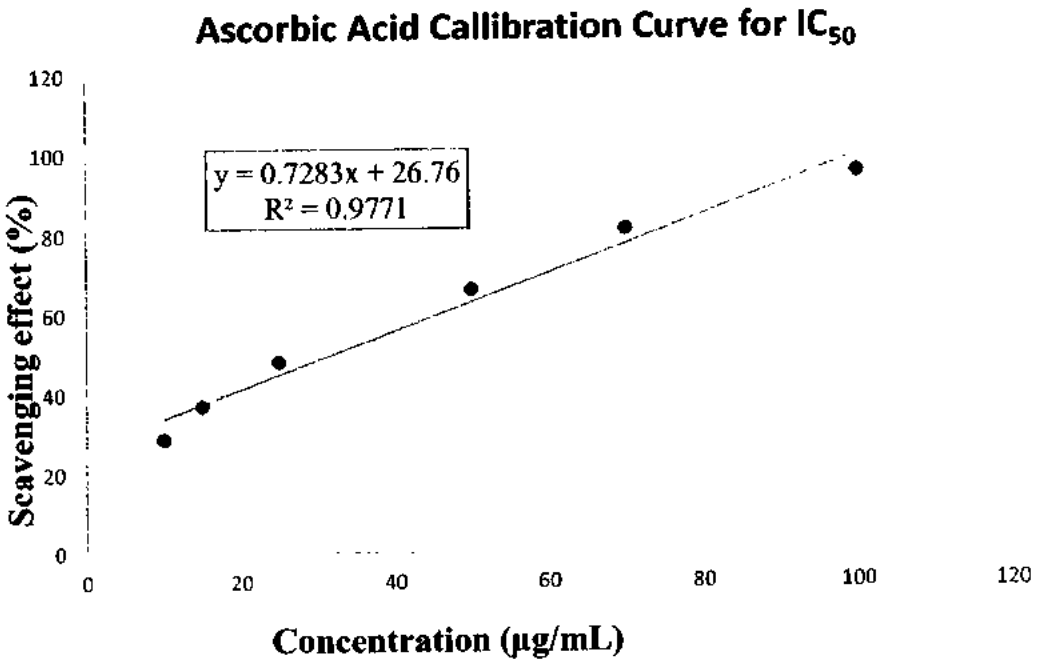


Figure 4.51: Ascorbic Acid (AA) Calibration Curve for IC_{50}

4.7. Phytochemical Analysis (Quantitative Analysis for Total Phenolic Contents and Total Flavonoids Contents)

4.7.1. Total Phenolic Contents (TPC)

Results of total phenolic contents (TPC) analysis of *G. wallichianum*, *E. parvifolia* and *A. alpinum* with crude methanolic extracts and more active fractionsare shown in table 4.10 below. TPC calculation was made from regression equation (Y= 0.0043X+0.0888, R^2= 0.9879) of standard gallic acid (GA) calibration curve as indicated in figure 4.52.TPC in GWME and its more active fractions (GWEF, GWBF and GWAF) are 237.094±0.695 (µg/mgGAE), 183.387±0.965 (µg/mgGAE), 05.579±1.645 (µg/mgGAE) and 154.640±2.104 (µg/mg GAE) respectively. The order of TPC in *G. wallichianum* is; GWME > GWEF > GWAF > GWBF. TPC of *E. parvifolia* (GWME) and its more active fractions (EPEF, EPBF and EPAF) are 242.581±0.708 (µg/mgGAE), 125.584±1.750 (µg/mg GAE), 68.322±1.520

(µg/mg GAE) and 142.430±1.645 (µg/mgGAE) respectively. The order of TPC in *E. parvifolia* is EPME> EPAF > EPEF > EPBF. TPC of AAME and its more active fractions are 192.892±0.814 (µg/mg GAE), 185.164±0.836 (µg/mgGAE), 109.433±1.771 (µg/mgGAE) and 165.304±0.708 (µg/mg GAE) respectively. TPC order in *AA* is AAME > AAEF > AAAF > AABF.

Table 4.10: TPC of Selected Active Plants Extracts/Fractions Calculated at Absorbance of 544 nm

Plant	Extract/ Fraction	Concentration of TPC (µg/mg GAE)
G. wallichianum	GWME	237.094±0.695
	GWEF	183.387±0.965
	GWBF	105.579±1.645
	GWAF	154.640±2.104
E. parvifolia	EPME	242.581±0.708
	EPEF	125.584±1.750
	EPBF	68.322±1.520
	EPAF	142.430±1.645
A. alpinum	AAME	192.892±0.814
	AAEF	185.164±0.836
	AABF	109.433±1.771
	AAAF	165.304±0.708

Results are expressed as mean+standard deviation (n=3)

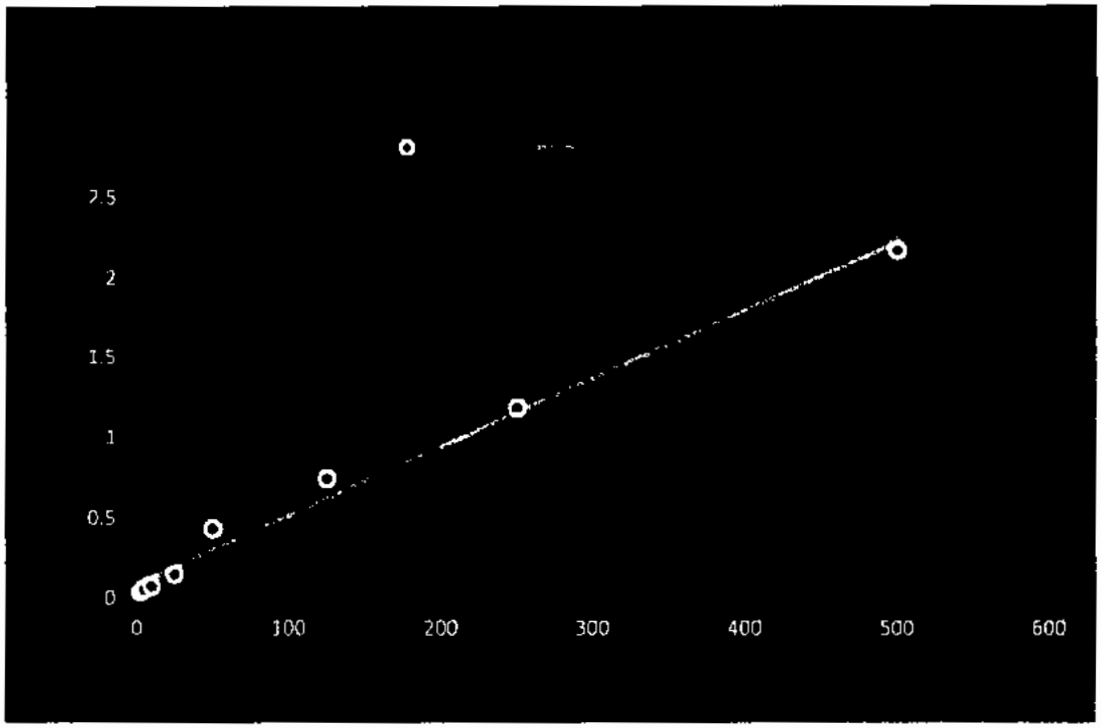


Figure 4.52: Gallic Acid (GA) Calibration Curve for TPC

4.7. 2. Total Flavonoid Contents (TFC)

Results of TFC of most active hepatoprotective plants (*G. wallichianum*, *E. parvifolia* and *A. alpinum*) are shown in table 4.11 below. Calculations of TFC was made from regression equation ($Y=0.0051X+0.0912$, $R^2=0.9964$) of standard quercetin calibration curve as indicated in figure 4.53. TFC in GWME and its active fractions (GWEF, GWBF and GWAF) are 95.843±0.582 (µg/mg QE), 118.171±0.194 (µg/mg QE), 84.129±0.112 (µg/mg QE) and 147.035±0.404 (µg/mg QE) respectively. Order for TFC is GWAF > GWEF > GWBF > GWME. In the case of *E. parvifolia* (EPME) and its active fractions (EPEF, EPBF and EPAF), the TFC are 196.027±0.194 (µg/mg QE), 113.705±0.388 (µg/mg QE), 45.233±0.624 (µg/mg QE) and 99.532±0.336 (µg/mg QE) respectively. Order for TFC in *E. parvifolia* is EPME > EPEF > EPAF > EPBF. For AAME and its active fractions (AAEF,

AABF and AAAF), TFC are 128.267±0.514 (µg/mg QE), 132.279±0.297 (µg/mg QE), 95.778±0.404 (µg/mg QE) and 150.659±0.489 (µg/mgQE) respectively. Order for TFC is AAAF > AAEF > AAME > AABF (table 4.11).

Table 4.11: TFC of Selected Active Plants Extracts/Fractions Calculated at Absorbance of 405 nm

Plant species	Extract/ fraction	Concentration of TFC (µg/mg QE)
G. wallichianum	GWME	95.843±0.582
	GWEF	118.171±0.194
	GWBF	84.129±0.112
	GWAF	147.035±0.404
E. parvifolia	EPME	196.027±0.194
	EPEF	113.705±0.388
	EPBF	45.233±0.624
	EPAF	99.532±0.336
A. alpinum	AAME	128.267±0.514
	AAEF	132.279±0.297
	AABF	95.778±0.404
	AAAF	150.659±0.489

Results are expressed as mean+standard deviation (n=3)

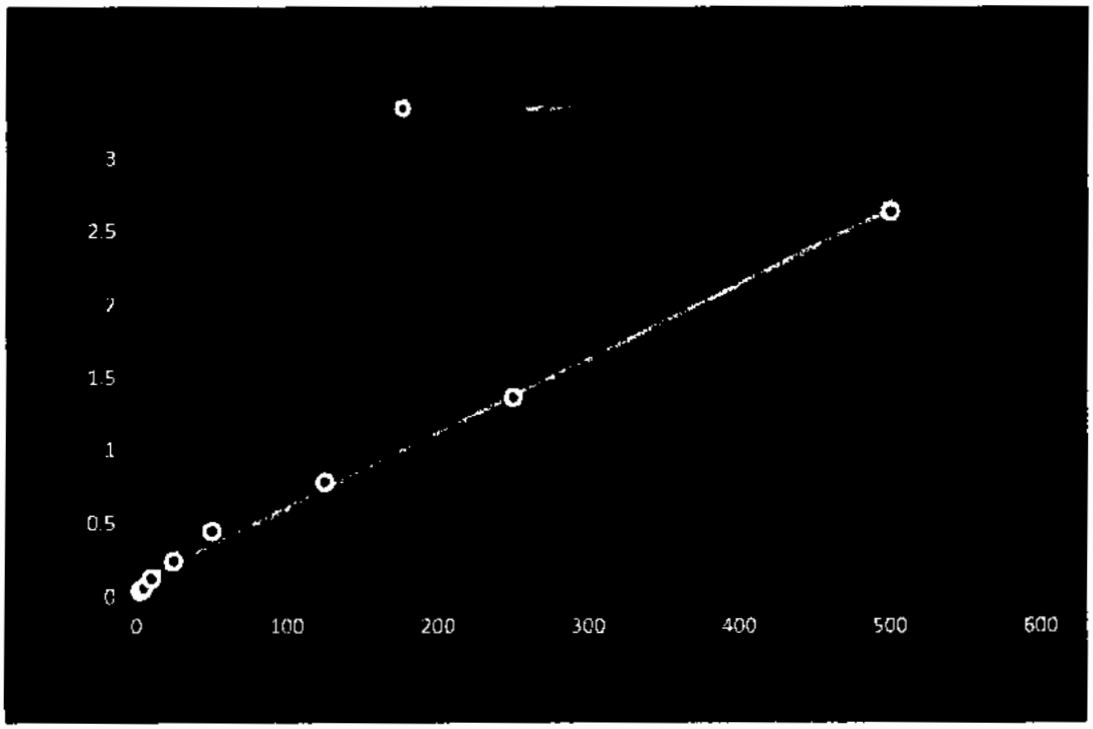


Figure 4.53: Quercetin Calibration Curve for TFC

4.8. Identification of Phytoconstituents of Short Listed Active Plants

4.8.1. HPLC Analysis of Methnolic Extract and More Active Fractions of Short Listed Active Plants

Detection of phenolics and flavonoids compounds by HPLC of three plants (*GW*, *EP* and *AA*) extracts in methanol and their active fractions was rendered quickly. Moreover, it also provided with good separation of the individual peaks as indicated in the chromatograms shown in figures (4.54-4.65) below respectively. Reference standard compounds used for identification of phenolics and flavonoids are shown by chromatogram in figure 4.54. Phenolics and flavonoids identified in these plants (*GW*, *EP* and *AA*) extracts are presented in tables 4.12 below. Reference standard of phenolics and flavoinoids (Gallic Acid, Caffeic Acid, Chlorogenic Acid, Epicatechin, Ferulic Acid, Hyperoside, Luteolin, Rutin, Fisetin, Apigenin-7- O-Glucoside, Naringenin, Benzene-Triol, Apigenin and Chrysin) were eluded

at 1.847, 10.070, 11.698, 15.256, 19.726, 29.110, 30.259, 30.893, 33.789, 35.810, 38.705, 42.768, 47.076 and 57.287 (min) respectively as represented by figure 4.54.

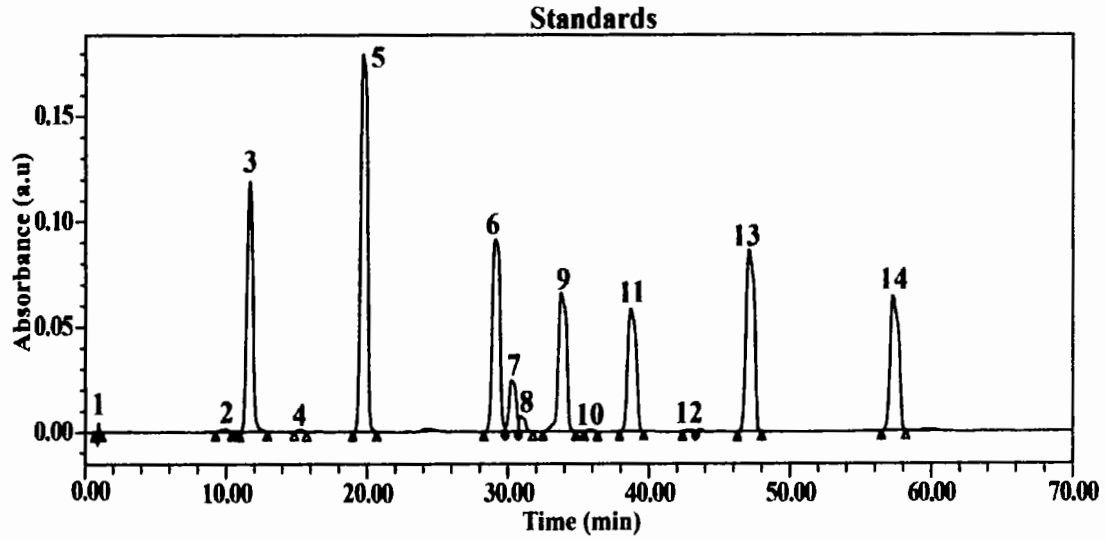


Figure 4.54: HPLC Chromatogram of Reference Standards For Flavonoids

Retention time of 1: Gallic Acid (1.847min), 2: Caffeic Acid (10.070min), 3: Chlorogenic Acid (11.698 min), 4: Epicatechin (15.256 min), 5: Ferulic Acid (19.726min), 6: Hyperoside (29.110 min), 7: Luteolin (30.259 min), 8: Rutin (30.893 min), 9: Fisetin (33.789 min), 10: Apigenin-7- O-Glucoside (35.810 min), 11: Naringenin (38.705 min),12: Benzene-Triol (42.768 min), 13: Apigenin (47.076 min) And 14: Chrysin (57.287 min).

4.8.1.1 HPLC Analysis of *G. wallichianum*

The presence of phenolic and flavonoids in GWME and its fractions i.e. GWEF, GWBF and GWAF with the peaks are indicated below in figures 4.55-4.58 respectively. Chromatogram of GW methanolic extract (GWME) revealed the ten peaks whose retention time was closely coincided with retention time of compound which showed two phenolic acids (Gallic acid and Ferulic acid), seven flavonoids (epicatechin, hyperoside, lutelin, rutin, fisetin, naringenin and apigenin) and one was benzene derived phenolic compound (benzene-triol) as shown in figure 4.55. The chromatograms of GWEF and GWBF showed fourteen peaks that were closely coincide to retention time of gallic acid, caffeic acid, chlorogenic acid,

epicatechin, ferulic acid, hyperoside, luteolin, rutin, fisetin, apigenin-7- o-glucoside, naringenin, benzene-triol, apigenin and chrysin as shown in figures 4.56 and 4.57 of which four were phenolic acid (gallic acid, caffeic acid, chlorogenic acid and ferulic acid), nine flavonoids (epicatechin, hyperoside, luteolin, rutin, fisetinn, apigenin-7-o-glucoside, naringenin, apigenin and chrysin) and one was benzene derived phenolic compound (benzene-triol).Similarly, from figure 4.58, it is evident that chromatograms of GWAF revealed the 13 peaks that were closely coincided with retention times of gallic acid, caffeic acid, chlorogenic acid, epicatechin, ferulic acid, hyperoside, luteolin, rutin, fisetin, apigenin-7- o-glucoside, naringenin, benzene-triol and apigenin respectively. In this plant, the higher number of phenolic compounds were identified in GWEF and GWBF (14 each) followed by GWAF (13) and GWME (10) as evident from the HPLC chromatograms (figure.4.55-4.58).

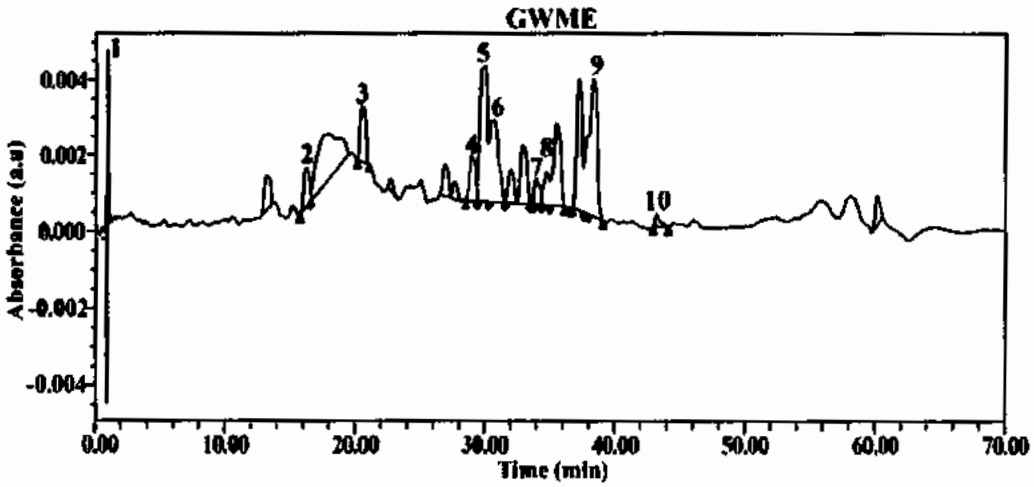


Figure 4.55: HPLC Chromatogram of *G. wallichianum* Methanolic Extract (GWME)

Peaks representing retention time to 1: gallic acid (0.996 min), 2: epicatechin (16.186 min), 3: ferulic acid (20.549 min), 4: hyperoside (28.798 min) 5: luteolin (30.013 min), 6: rutin (30.720 min), 7: fisetin (33.733 min), 8: apigenin-7- o-glucoside (34.753 min), 9: naringenin (38.327 min) and 10: benzene-triol (43.209 min).

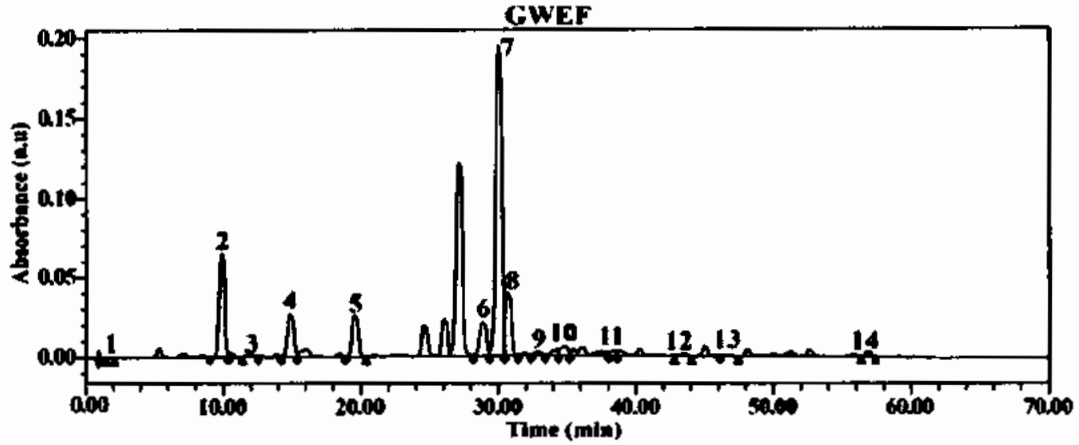


Figure 4.56: HPLC Chromatogram of *G. wallichianum* Ethyl Acetate Fraction (GWEF)

Peaks representing retention time to 1: gallic acid (1.813 min), 2: caffeic acid (9.893 min), 3: chlorogenic acid (12.208 min), 4: epicatechin (14.871 min), 5: ferulic acid (19.545 min), 6: hyperoside (28.582 min), 7: luteolin (30.012 min), 8: rutin (30.664 min), 9: fisetin (32.904 min), 10: apigenin-7- o-glucoside (34.214 min), 11: naringenin (38.857 min),12: benzene-triol (43.250 min), 13: apigenin (46.779 min) and 14: chrysin (56.859 min).

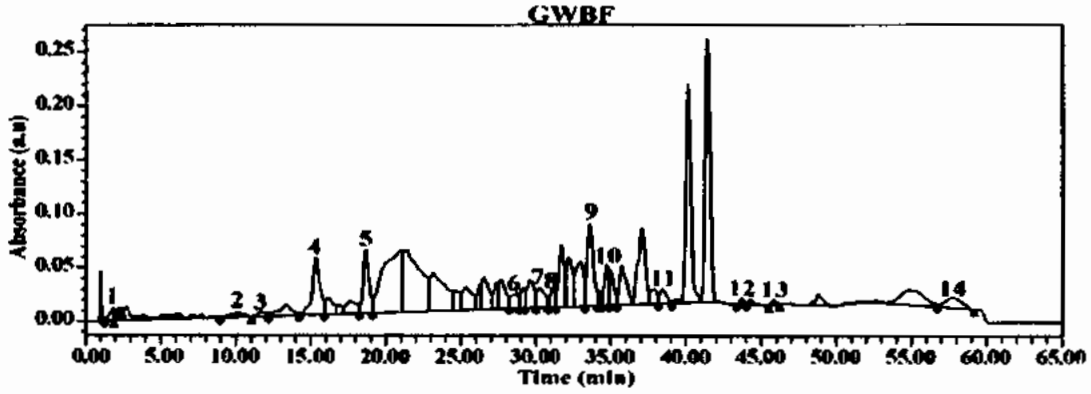


Figure 4.57: HPLC Chromatogram of *G. wallichianum* n-Butanol Fraction (GWBF)

Peaks representing retention time to 1: gallic acid (1.827 min), 2: caffeic acid (10.096 min), 3: chlorogenic acid (11.682 min), 4: epicatechin (15.347 min), 5: ferulic acid (18.654 min), 6: hyperoside (28.827 min), 7: luteolin (30.211 min), 8: rutin (31.152 min), 9: fisetin (33.580 min), 10: apigenin-7- o-glucoside (34.239 min), 11: naringenin (34.435 min),12: benzene-triol (43.215 min), 13: apigenin (46.977 min) and 14: chrysin (57.733 min).

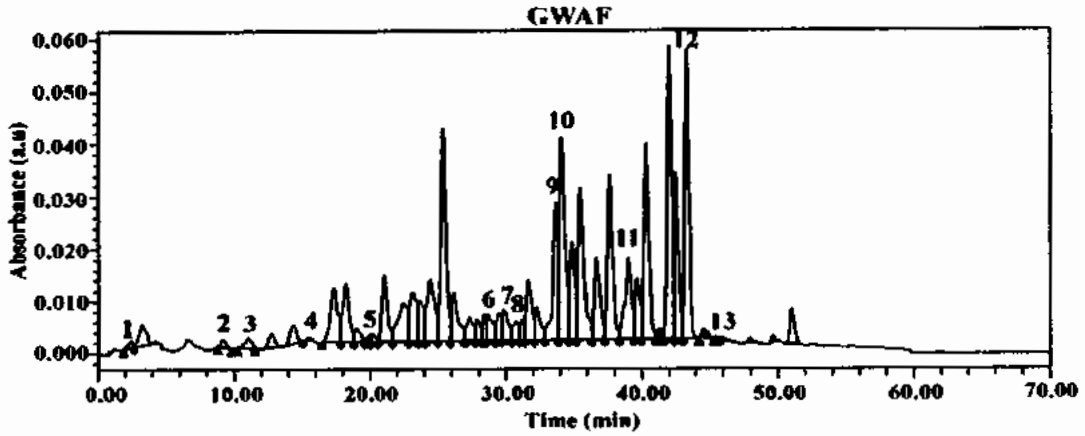


Figure 4.58: HPLC Chromatogram of *G. wallichianum* Aqueous Fraction (GWAF)

Peaks representing retention time to 1: gallic acid (2.468 min), 2: caffeic acid (9.271 min), 3: chlorogenic acid (11.082 min), 4: epicatechin (15.525 min), 5: ferulic acid (2.033 min), 6: hyperoside (28.705 min), 7: luteolin (39.92 min), 8: rutin (30.784 min), 9: fisetin (33.720 min), 10: apigenin-7- o-glucoside (34.137 min), 11: naringenin (39.050 min),12: benzene-triol (43.339 min) and 13: apigenin (45.98 min).

4.8.1.2. HPLC Analysis of *E. parvifolia*

Presence of phenolic and flavonoids in *EP* fruits (EPME and fractions i.e. EPEF, and EPAF fractions) is indicated with peaks in figures 4.59-4.61 respectively. HPLC chromatogram of crude methanolic extract of *E. parvifolia* (EPME) showed nine peaks whose retention time was closely coincided with peaks of of gallic acid, epicatechin, ferulic acid, hyperoside, luteolin, rutin, apigenin-7- o-glucoside, benzene-triol and apigenin as indicated in figure 4.59. Chromatogram of EPEF with twelve peaks of which closely coincided with gallic acid, caffeic acid, chlorogenic acid, epicatechin, ferulic acid, hyperoside, luteolin, rutin , fisetin, naringenin, benzene-triol and chrysin (figure 4.60) Similarly from figure 4.61, it is shown that chromatograms of EPAF showed 10 peaks coincided to gallic acid, caffeic acid, ferulic acid, hyperoside, luteolin, apigenin-7- o-glucoside, naringenin, benzene-triol, apigenin and chrysin. Order for presence of flavonoids is; ethyl acetate fraction (12) > aqueous fraction (10) >crude methanolic fraction (9) as evident from the HPLC chromatograms (figure 4.59-4.61).

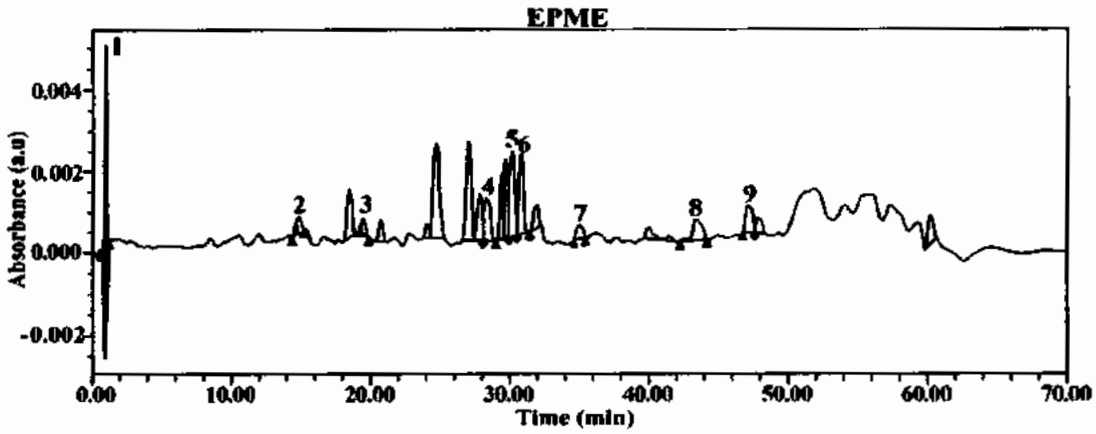


Figure 4.59: HPLC Chromatogram of *E. parvifolia* Methanolic Extract (EPME)

Peaks indicating retention time to 1: gallic acid (0.974 min), 2: epicatechin (14.806 min), 3: ferulic acid (19.467 min), 4: hyperoside (28.294 min), 5: luteolin (30.164 min), 6: rutin (30.768 min), 7: apigenin-7- o-glucoside (34.979 min), 8: benzene-triol (43.393 min) and 9: apigenin (47.073 min).

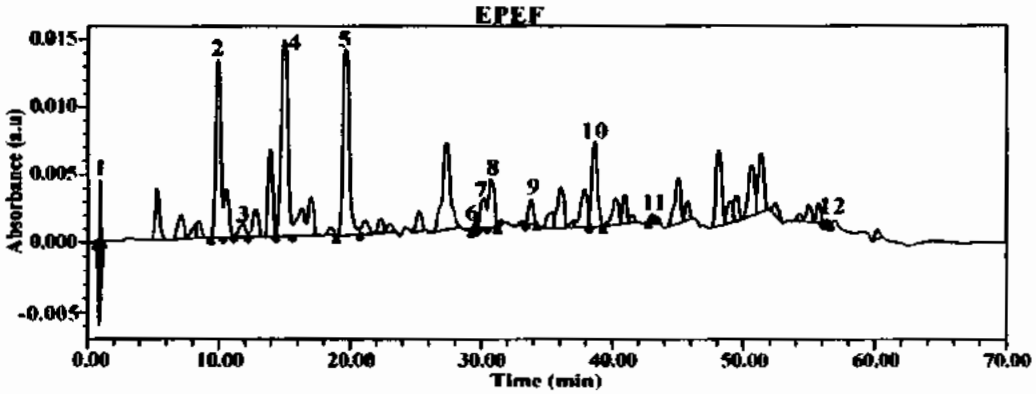


Figure 4.60: HPLC Chromatogram of *E. parvifolia* Ethyl Acetate Fraction (EPEF)

Peaks indicating retention time to 1: gallic acid (0.968 min), 2: caffeic acid (9.961 min), 3: chlorogenic acid (11.792 min), 4: epicatechin (15.008 min), 5: ferulic acid (19.660 min), 6: hyperoside (29.626 min), 7: luteolin (30.232 min), 8: rutin (30.748 min), 9: fisetin (33.808 min), 10: naringenin (38.691 min),12: benzene-triol (43.00 min) and 13: chrysin (56.330 min).

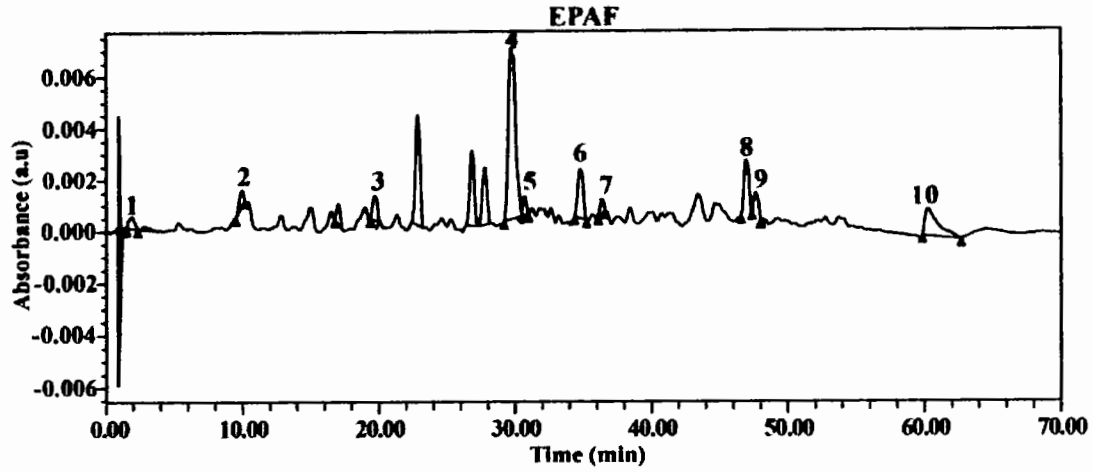


Figure 4.61: HPLC Chromatogram of *E. parvifolia* Aqueous Fraction (EPAF)

Peaks representing retention time to 1: gallic acid (1.910 min), 2: caffeic acid (9.933 min), 3: ferulic acid (19.728 min), 4: hyperoside (29.713 min), 5: luteolin (30.713 min), 6: apigenin-7- o-glucoside (34.804 min), 7: naringenin (36.402 min), 8: benzene-triol (46.961 min), 9: apigenin (47.636 min) and 10: chrysin (60.250 min).

4.8.1.3. HPLC Analysis of *A. alpinum*

The results of HPLC of AAME and its fractions (AAEF, AABF and AAAF) with the chromatograms peaks are indicated infigures 4.62-4.65 respectively. AAME chromatogram indicated 7 peaks which coinsided with gallic acid, chlorogenic acid , epicatechin, rutin, apigenin-7- o-glucoside, naringenin and chrysin (figur 4.62). AAEF showed ten peaks which coincided closely with gallic acid, caffeic acid, chlorogenic acid, epicatechin, ferulic acid, luteolin, rutin, apigenin-7-o-glucoside, naringenin and chrysin (figure 4.63) respectively. Fourteen phenolics compounds were detected in AABF whose peaks coincided closely with gallic acid, caffeic acid, chlorogenic acid, epicatechin, ferulic acid, hyperoside, luteolin, rutin, fisetin, apigenin-7- o-glucoside, naringenin, benzene-triol, apigenin and chrysin (figure 4.64) respectively. From figure 4.65, it is clearly indicated that AAAF have the presence of 12 compounds that coincided with caffeic acid, chlorogenic acid, epicatechin, ferulic acid, hyperoside, luteolin, rutin, apigenin-7- o-glucoside, naringenin, benzene-triol, apigenin and chrysin. Flavonoids compounds with higher number is identified in AABF (14)

followed by AAAF (12), AAEF (10) and AAME (7) as shown by HPLC chromatograms (figures 4.62-4.65).

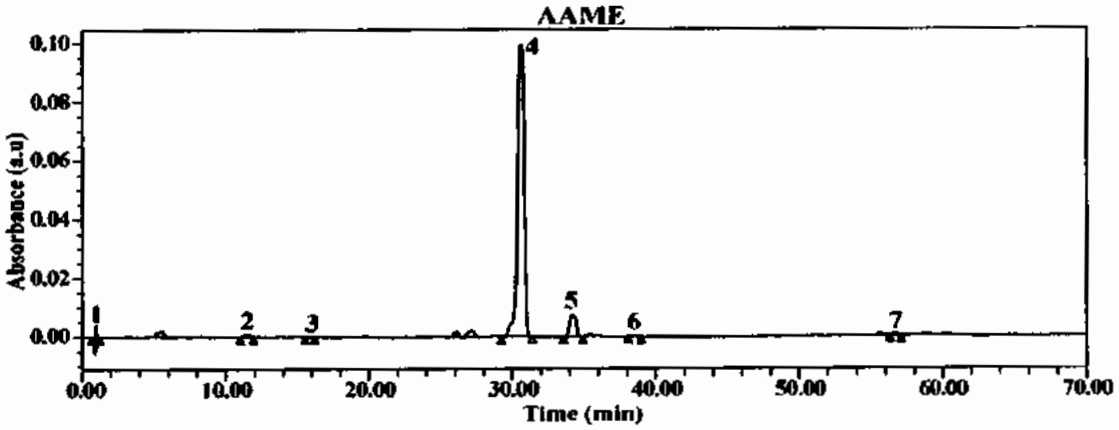


Figure 4.62: HPLC Chromatogram of *AAlpinum* Methanolic Extract (AAME)

Peaks representing retention time to 1: gallic acid (0.960 min), 2: chlorogenic acid (11.479 min), 3: epicatechin (15.877 min), 4: rutin (30.591 min), 5: apigenin-7- o-glucoside (34.207 min), 6: naringenin (38.467 min) and 7: chrysin (56.663 min).

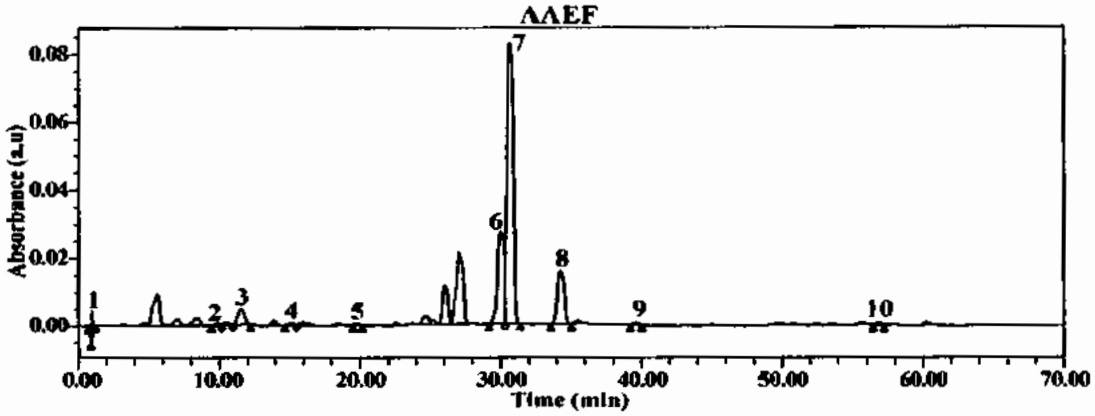


Figure 4.63: HPLC Chromatogram of *A. Alpinum* Ethyl Acetate Fraction (AAEF)

Peaks representing retention time to1: gallic acid (0.970 min), 2: caffeic acid (9.888 min), 3: chlorogenic acid (11.550 min), 4: epicatechin (15.113 min), 5: ferulic acid (19.763 min), 6:luteolin (29.988 min), 7:rutin (30.660 min), 8: apigenin-7-o glucoside (34.263 min), 9:naringenin (39.615 min) and 10: chrysin (56.784 min).

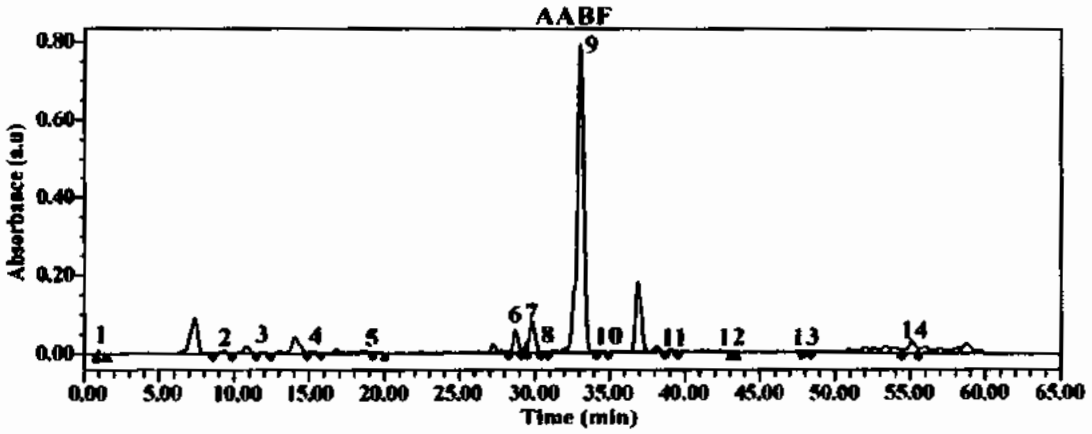


Figure 4.64: HPLC Chromatogram of *A. Alpinum* n-Butanol Fraction (AABF)

In figure 4.64 above, peaks representing similar retention time to 1: gallic acid (1.263 min), 2: caffeic acid (9.366 min), 3: chlorogenic acid (11.870 min), 4: epicatechin (15.221 min), 5: ferulic acid (19.438 min), 6: hyperoside (28.723 min), 7: luteolin (29.822 min), 8: rutin (30.508 min), 9: fisetin (33.043 min), 10:apigenin-7- o-glucoside (34.444 min), 11: naringenin (39.054 min), 12: benzene-triol (43.104 min), 13: apigenin (47.538 min) and 14: chrysin (56.962 min).

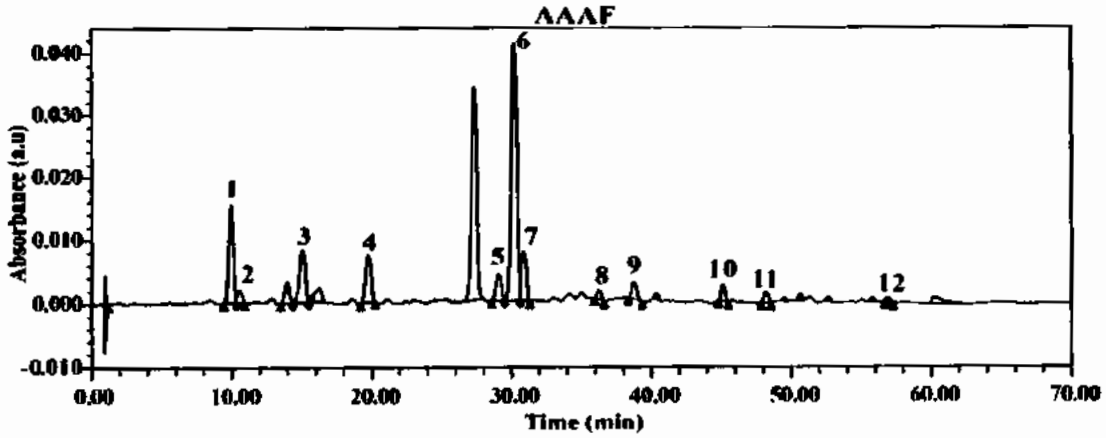


Figure 4.65: HPLC Chromatogram of *A. Alpinum* Aqueous Fraction (AAAF)

Peaks representing similar retention time to 1: caffeic acid (9.969 min), 2: chlorogenic acid (10.569 min), 3: epicatechin (15.075 min), 4: ferulic acid (19.765 min), 5: hyperoside (29.076 min), 6: luteolin (30.208 min), 7: rutin (30.891 min), 8: apigenin-7- o-glucoside (36.265 min), 9: naringenin (38.781 min), 10: benzene-triol (48.213 min),11: apigenin (48.213 min) and 12: chrysin (56.920 min).

Table 4.12: Compounds Identifiedby HPLC in Selected Active Plants

Standards	R-time (min)	*G. wallichianum* Extracts				*E. parvifolia* Extracts			*A. alpinum* Extracts			
		GW ME	GW EF	GW BF	GW AF	EP ME	EP EF	EP AF	AA ME	AA EF	AA BF	AA AF
Gallic Acid	1.847	+	+	+	+	+	+	+	+	+	+	−
Caffeic Acid	10.07	−	+	+	+	−	+	+	−	+	+	+
Chlorogenic Acid	11.698	−	+	+	+	−	+	−	+	+	+	+
Apicatechin	15.256	+	+	+	+	+	+	−	+	+	+	+
Ferulic Acid	19.726	+	+	+	+	+	+	+	−	+	+	+
Hyperoside	29.110	+	+	+	+	+	+	+	−	−	+	+
Luteolin	30.259	+	+	+	+	+	+	+	−	+	+	+
Rutin	30.893	+	+	+	+	+	+	−	+	+	+	+
Fisetin	33.789	+	+	+	+	−	+	−	−	−	+	−
Apigenin-7-o glucoside	35.810	+	+	+	+	+	−	+	+	+	+	+
Naringenin	38.705	+	+	+	+	−	+	+	+	+	+	+
Benzotriol	42.768	+	+	+	+	+	+	+	−	−	+	+
Apigenin	47.076	−	+	+	+	+	−	+	−	−	+	+
Chrysin	57.287	−	+	+	−	−	+	+	+	+	+	+

4.8.2. LCMS Analysis of Short Listed Active Hepatoprotective Plants

Compounds identified by LCMS QTOF analysis of *G. wallichianum*, *E. parvifilia* and *A. alpinum* are represented in the tables 4.13-4.15 below respectively. Total sixteen compounds (syringic acid, gallic acid, gallic acid dimer, protocatechuic, kamferol, quercitrin, rutin, quercetine glucoside (hyperoside), apigenin, epicatechin, catechin, quinic acid, chlorogenic acid, quercetine, caffeic acid and vanillic acid) were identified in three plants. The identified polyphenolic compounds in methanolic extract, aqueous fraction and ethyl acetate fraction of each plant are given seperately in the following manner.

4.8.2.1. LCMS Analysis of *G. wallichianum*

The poly phenolic compounds identified by LCMS QTOF analysis of *G. wallichianum* are shown in the table 4.13 below. *G. wallichianum* methanolic extract (GWME) showed the presence of eight compounds (syringic acid, gallic acid dimer, protocatechuic acid, kaempferol, quercitrin, quercitine glucoside (hyperoside), apigenin (trc)and quinic acid. Eleven compounds (i. e. syringic acid, gallic acid, gallic acid dimer, protocatechuic acid, kamferol, quercitrin, rutin, quercitine glucoside (hyperoside), apigenin (trc), catechin and quinic acid) were identified in aqueous fraction of *G. wallichianum* (GWAF). In the ethyl acetate fraction of *G. wallichianum* (GWEF), nine compounds (e. g syringic acid, gallic acid, gallic acid dimer, protocatechuic acid, kamferol, quercitrin, apigenine (trc), epicatechin and quinic acid) were identified. The order of presence of compounds is GWAF (11) > GWEF (9) > GWME (7) respectively.

Table 4.13: LCMS Analysis of ME of *G. wallichianum* and Most Active Fractions

Mode	Rt (min)	Mol. Formula	m/z Experimental	m/z Calculated	Delta (mmu)	RDB	Proposed compound	GW ME	GW AF	GW EF
[M-H]⁻	19.6	$C_9H_{10}O_5$	197.045	197.046	5.29	5	Syringic acid	+	+	+
[M-H]⁻	6.9	$C_7H_6O_5$	169.016	169.014	-9.72	5	Gallic acid	-	+	+
[M-H]⁻	7.3	$C_{14}H_{12}O_{10}$	339.034	339.036	4.91	9	Gallic acid dimer	+	+	+
[M-H]⁻	12.7	$C_7H_6O_4$	153.019	153.019	3.46	5	Protocatechuic acid	+	+	+
[M-H]⁻	25.5	$C_{15}H_{10}O_6$	285.041	285.041	-2.23	11	Kaempferol	+	+	+
[M-H]⁻	20.2	$C_{21}H_{20}O_{11}$	447.09	447.093	7.33	12	Quercitrin	+	+	+
[M-H]⁻	19.7	$C_{27}H_{30}O_{16}$	609.142	609.146	6.24	13	Rutin	-	+	-
[M-H]⁻	19.6	$C_{21}H_{20}O_{12}$	463.09	463.095	-1.5	12	Hyperoside	+	+	-
[M-H]⁻	34.8	$C_{15}H_{10}O_5$	269.047	269.046	-6.12	11	Apigenin	tr	tr	tr
[M-H]⁻	16.6	$C_{15}H_{14}O_6$	289.074	289.072	-6.68	9	Epicatechin	-	-	+
[M-H]⁻	14.5	$C_{15}H_{14}O_6$	289.071	289.072	2.63	9	Catechin	-	+	-
[M-H]⁻	2.5	$C_7H_{12}O_6$	191.057	191.056	-5.15	2	Quinic acid	+	+	+
[M-H]⁻	19	$C_{16}H_{18}O_9$	353.084	353.088	10.47	8	Chlorogenic acid	-	-	-
[M-H]⁻	25.2	$C_{15}H_{10}O_7$	301.039	301.035	-11.67	11	Quercetin	-	-	-
[M-H]⁻	15.7	$C_9H_8O_4$	179.034	179.035	6.57	6	Caffeic acid	-	-	-
[M-H]⁻	6.017	$C_8H_8O_4$	167.033	167.035	11.8	5	Vanillic acid	-	-	-

4.8.2.2. LCMS Analysis of *E. parvifolia*

LMS QTOF analysis of *E. parvifolia* revealed the presence of seven phenolic compounds (gallic acid dimer, protocatechuic acid. kaempferol, quercitrin, quercetine glucoside (hyperoside), apigenin (trc) and quinic acid) in *E. parvifolia* methanolic extract (EPME) while four compounds (e.g. gallic acid, gallic acid dimer, apigenin(trc) and protocatechuic acid were identified in the EPAF. LCMS-QTOF analysis of EPEF showed the presence of twelve phenolic compounds e. g gallic acid, gallic acid dimer, protocatechuic acid, quercitrin, quercetine glucoside (hyperoside), apigenin (trc), epicatechin, catechin,

quenic acid, chlorogenic acid, quercetine and caffeic acid. The identified compounds are represented in table 4.14 below. The order of presence of compounds is EPEF (12) > EPME (7) > EPAF (4) respectively.

Table 4.14: LCMS Analysis of ME of *E. parvifolia* and Most Active Fractions

Mode	Rt (min)	Mol. Formula	m/z Experimental	m/z Calculated	Delta (mmu)	RDB	Proposed Compound	EP ME	EP AF	EP EF
[M-H]⁻	19.6	$C_9H_{10}O_5$	197.0445	197.0455	5.29	5	Syringic acid	-	-	-
[M-H]⁻	6.9	$C_7H_6O_5$	169.0159	169.0142	-9.72	5	Gallic acid	-	+	+
[M-H]⁻	7.3	$C_{14}H_{12}O_{10}$	339.0341	339.0358	4.91	9	Gallic acid dimer	+	+	+
[M-H]⁻	12.7	$C_7H_6O_4$	153.0188	153.0193	3.46	5	Protocatechuic acid	+	+	+
[M-H]⁻	25.5	$C_{15}H_{10}O_6$	285.0411	285.0405	-2.23	11	Kaempferol	+	-	-
[M-H]⁻	20.2	$C_{21}H_{20}O_{11}$	447.09	447.0933	7.33	12	Quercitrin	+	-	+
[M-H]⁻	19.7	$C_{27}H_{30}O_{16}$	609.1423	609.1461	6.24	13	Rutin	-	-	-
[M-H]⁻	19.6	$C_{21}H_{20}O_{12}$	463.0899	463.0949	-1.5	12	Hyperoside	+	-	+
[M-H]⁻	34.8	$C15H_{10}O_5$	269.0472	269.0455	-6.12	11	Apigenin	tr	tr	tr
[M-H]⁻	16.6	$C_{15}H_{14}O_6$	289.0737	289.0718	-6.68	9	Epicatechin	-	-	+
[M-H]⁻	14.5	$C_{15}H_{14}O_6$	289.071	289.0718	2.63	9	Catechin	-	-	+
[M-H]⁻	2.5	$C_7H_{12}O_6$	191.0571	191.0561	-5.15	2	Quinic acid	+	-	+
[M-H]⁻	19	$C_{16}H_{18}O_9$	353.0841	353.0878	10.47	8	Chlorogenic acid	-	-	+
[M-H]⁻	25.2	$C_{15}H_{10}O_7$	301.0389	301.0354	-11.67	11	Quercetin	-	-	+
[M-H]⁻	15.7	$C_9H_8O_4$	179.0338	179.035	6.57	6	Caffeic acid	-	-	+
[M-H]⁻	6.017	$C_8H_8O_4$	167.033	167.035	11.8	5	Vanillic acid	-	-	-

4.8.2.3. LCMS Analysis of *A. alpinum*

Compounds identified from *A. alpinum* are shown in table 4.15 below. LCMS analysis of *A. alpinum* methanolic extract (AAME) revealed the presence of nine phenolic compounds (gallic acid dimer, protocatechuic acid, kaempferol (trc), quercitrin, rutin, quercetine glucoside (hyperoside), apigenin, quinic acid and chlorogenic acid. In aqueous

fraction of *A. alpinum* (AAAF) fifteen compounds (syringic acid, gallic acid, gallic acid dimer, protocatechuic, kaempferol, quercitrin, rutin, quercetin glucoside (hyperoside), apigenin(trc), epicatechin, catechin, chlorogenic acid, quercetine, caffeic acid and vanillic acid) were identified by LCMS analysis. Fifteen phenolic compounds (syringic acid, gallic acid, gallic acid dimer, protocatechuic, kaempferol, quercitrin, rutin, quercetine glucoside (hyproside), apigenin, epicatechin, catechin, quinic acid, quercetin, caffeic acid and vanillic acid) were identified from ethyl acetate fraction of *A. alpinum* (AAEF). The order of presence of phenolic compounds is AAAF (15) =AAEF (15)> AAME (9) respectively.

Table 4.15: LCMS Analysis of ME of *A. alpinum* and Most Active Fractions

Mode	Rt (min)	Mol. Formula	m/z Experimental	m/z Calculated	Delta (mmu)	RDB	Proposed Compound	AA ME	AA AF	AA EF
[M-H]⁻	19.6	$C_9H_{10}O_5$	197.0445	197.0455	5.29	5	Syringic acid	-	+	+
[M-H]⁻	6.9	$C_7H_6O_5$	169.0159	169.0142	-9.72	5	Gallic acid	-	+	+
[M-H]⁻	7.3	$C_{14}H_{12}O_{10}$	339.0341	339.0358	4.91	9	Gallic acid dimer	+	+	+
[M-H]⁻	12.7	$C_7H_6O_4$	153.0188	153.0193	3.46	5	Protocatechuic acid	+	+	+
[M-H]⁻	25.5	$C_{15}H_{10}O_6$	285.0411	285.0405	-2.23	11	Kaempferol	trc	+	+
[M-H]⁻	20.2	$C_{21}H_{20}O_{11}$	447.09	447.0933	7.33	12	Quercitrin	+	+	+
[M-H]⁻	19.7	$C_{27}H_{30}O_{16}$	609.1423	609.1461	6.24	13	Rutin	+	+	+
[M-H]⁻	19.6	$C_{21}H_{20}O_{12}$	463.0899	463.0949	-1.5	12	Hyperoside	+	+	+
[M-H]⁻	34.8	$C_{15}H_{10}O_5$	269.0472	269.0455	-6.12	11	Apigenin	+	trc	+
[M-H]⁻	16.6	$C_{15}H_{14}O_6$	289.0737	289.0718	-6.68	9	Epicatechin	-	+	+
[M-H]⁻	14.5	$C_{15}H_{14}O_6$	289.071	289.0718	2.63	9	Catechin	-	+	+
[M-H]⁻	2.5	$C_7H_{12}O_6$	191.0571	191.0561	-5.15	2	Quinic acid	+	-	+
[M-H]⁻	19	$C_{16}H_{18}O_9$	353.0841	353.0878	10.47	8	Chlorogenic acid	+	+	-
[M-H]⁻	25.2	$C_{15}H_{10}O_7$	301.0389	301.0354	-11.67	11	Quercetin	-	+	+
[M-H]⁻	15.7	$C_9H_8O_4$	179.0338	179.035	6.57	6	Caffeic acid	-	+	+
[M-H]⁻	6.017	$C_8H_8O_4$	167.033	167.035	11.8	5	Vanillic acid	-	+	+

Most abundant compounds i.e. Rutin and quercetin were found in the extract and MS data for these compounds is presented together with other fragmentation pictures below (figure 4.66 and 4.67).

Table 4.16 Limits of Detection Obtained for the Analysed Samples During LCMS

LOD		LOQ (3xLOD)	
Quercitrin	6.4 ng/mL	quercitrin	19.2 ng/mL

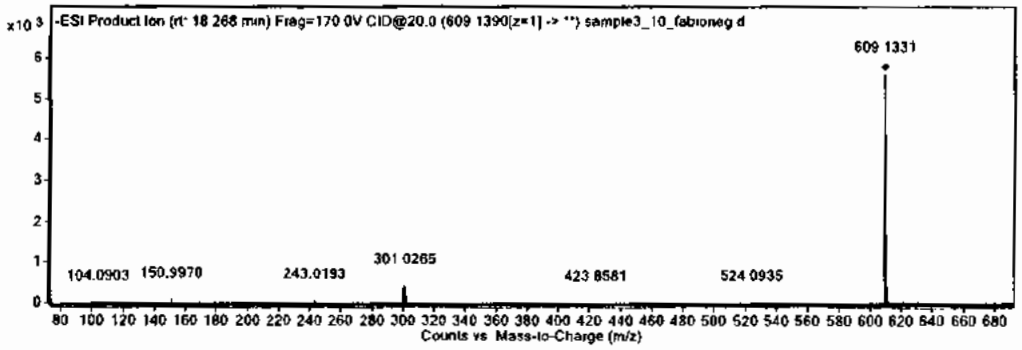


Figure 4.66: MS/MS Spectrum of Rutin Showing Dominant Fragment of Quercetin (301 m/z)

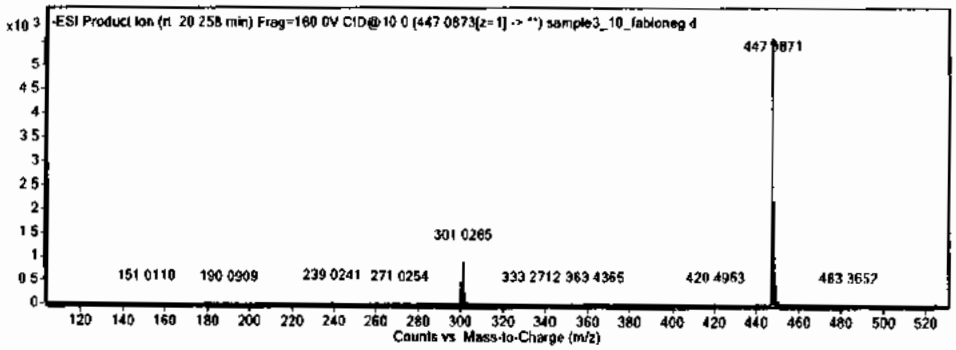


Figure 4.67: Fragmentation of Quercitrin Showing Derivative of Quercetin

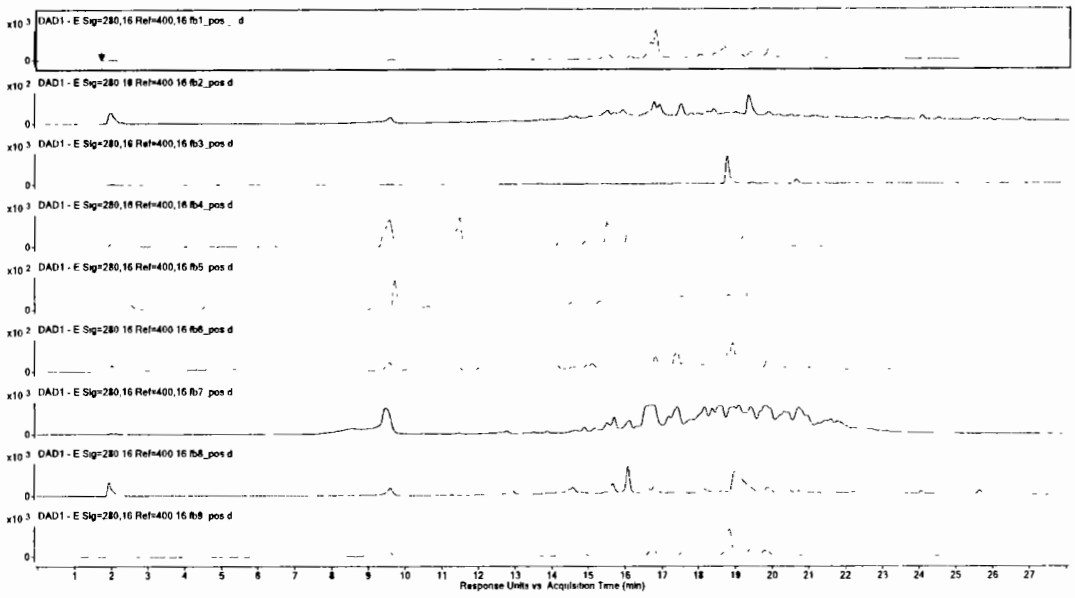


Figure 4.68: Metabolic Profiles of All Studied Samples Recorded in 280 nm

4.8.2.4. MS/MS Spectra of Identified Compounds

The MS/MS spectra of identified compounds (gallic acid, protocatechuic acid, kaempferol quercitrin, rutin, apicatechin, catechin, quinic acid, chlorogenic acid, quercetin, caffeic acid andvanillic acid) from methanolic extract and most active fractions of three most active hepatoprotective plants obtained by LCMS analysis are represented below in figures 4.69-4.80 respectively. Figure 4.69 shows MS-spectra obtained in negative ionization mode, which shows deprotonated pseudomolecular ion at m/z 169.0130, with major fragments at m/z 125.0228. As m/z 169.0130 is in good agreement with monoisotopic mass of gallic acid, this compound is therefore tentatively identified as gallic acid. Figure 4.70 shows MS-spectra obtained in negative ionization mode, which shows deprotonated pseudo molecular ion with m/z 153.0195, having major fragments at m/z 135.0538 and 109.0289. Fragment signals at m/z 109 [(M-H)-44], shows loss of carboxylic acid moiety, therefore this compound is tentatively identified as protocatechuic acid. Figure 4.71 shows MS-spectra obtained in negative ionization mode, which shows deprotonated pseudomolecular ion with m/z 285.0475, having major fragments at m/z 267.047, 209,320, 153.0041 and 109.0163.

Keeping in view the fragments and the pseudomolecular ion, this compound is tentatively identified as kaempferol. Figure 4.72 shows MS-spectra obtained in negative ionization mode, which shows deprotonated pseudo molecular ion at m/z 447.0953, with major fragments on m/z 357.0595, 327.0486 and 253.0335. Fragment signals on m/z 357 [(M-H)-90] and 327 [(M-H)-120], show loss of 90 amu and 120 amu fragments, which is a characteristic feature of glycosyl flavonoids. Keeping in view the fragments and the pseudomolecular ion, this compound is tentatively identified as quercitrin. Figure 4.73 shows MS-spectra obtained in negative ionization mode at 18.686 Rt, which shows deprotonated pseudo molecular ion at m/z 609.1380, with fragment signals at m/z 464.0604, 301.0193 and 178.9953. Fragment signal at m/z 301 shows quercetin, while fragment signal at m/z 464 shows loss of rhamnosyl moiety. As m/z 609.1380 is in good agreement with monoisotopic mass of rutin, therefore this compound is tentatively identified as rutin.

Figure 4.74 shows MS-spectra obtained in negative ionization mode, which shows deprotonated pseudomolecular ion at m/z 289.0676, with major fragments at m/z 271.0624, 245.0815, 209.996, 178.9925 and 125.0265. Fragment signal at m/z 245 shows loss of carboxylic acid moiety (44 amu). Keeping in view the fragments and the pseudomolecular ion, this compound is tentatively identified as epicatechin. Figure 4.75 shows MS-spectra obtained in negative ionization mode, which shows deprotonated pseudomolecular ion at m/z 289.0676, with major fragments at m/z 245.0815, 203.0665 and 179.0317. Fragment signal at m/z 245 shows loss of carboxylic acid moiety (44amu). Keeping in view the fragments and the pseudomolecular ion, this compound is tentatively identified as catechin. Figure 4.76 shows MS-spectra obtained in negative ionization mode, which shows deprotonated pseudomolecular ion at m/z 191.0080, with fragment signals at m/z 147.0574, 129.0127 and 111.0016. Fragment signal at m/z 147 shows loss of carboxylic acid moiety (44 amu). Therefore, this compound is tentatively identified as quinic acid. Figure 4.77 shows MS-spectra obtained in negative ionization mode, which shows deprotonated pseudomolecular ion at m/z 353.088, with fragment signals at m/z 191.055, 179.033, 161.020 and 135.044. Fragment signal at m/z 191 shows quinic acid moiety, while fragment signals at m/z 179 and m/z 135 indicate presence of caffeic acid moiety. As m/z 353.0881 is in good agreement with m/z 353.0878, therefore this compound is tentatively identified as chlorogenic acid (caffeoyl

quinic acid). Figure 4.78 shows MS-spectra obtained in negative ionization mode at retention time (Rt)7.809(min), which shows deprotonated pseudomolecular ion at m/z 301.0546, with fragment signals at m/z 283.0378, 254.84 and 168.0041. This compound is tentatively identified as quercetin. Figure 4.79 shows MS-spectra obtained in negative ionization mode, which shows deprotonated pseudomolecular ion at m/z 180.0343, with fragment signals at m/z 135.0420. Fragment signal at m/z 135 shows loss of carboxylic acid moiety (44 amu). Therefore, this compound is tentatively identified as caffeic acid. Figure 4.80 shows MS-spectra obtained in negative ionization mode, which shows deprotonated pseudomolecular ion at m/z 167.0338, with fragment signals at m/z 149.0219, 123.0453 and 106.0355. Fragment signal at m/z 149 shows tartaric acid moiety, while fragment signals at m/z 123 and m/z 106 indicate presence of benzoic acid and benzoyl moiety. As m/z 167.0338 is in good agreement with m/z 167.0330, therefore this compound is tentatively identified as vanillic acid.

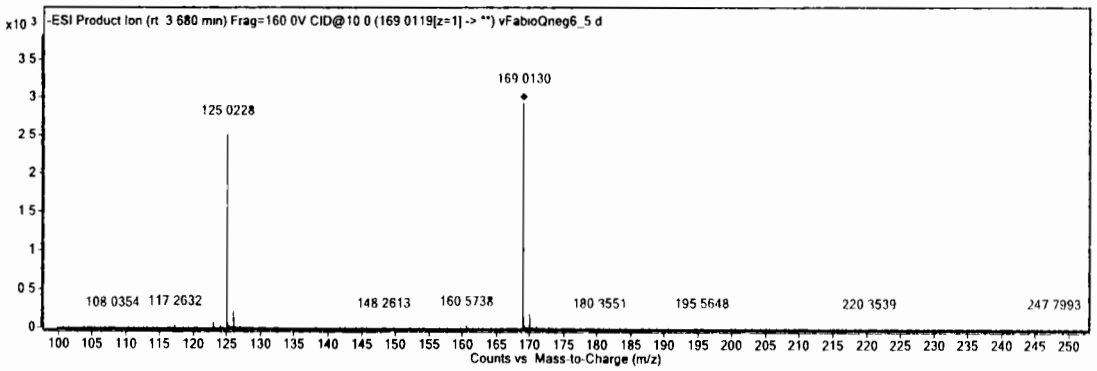


Figure 4.69: MS/MS Spectrum of Gallic Acid

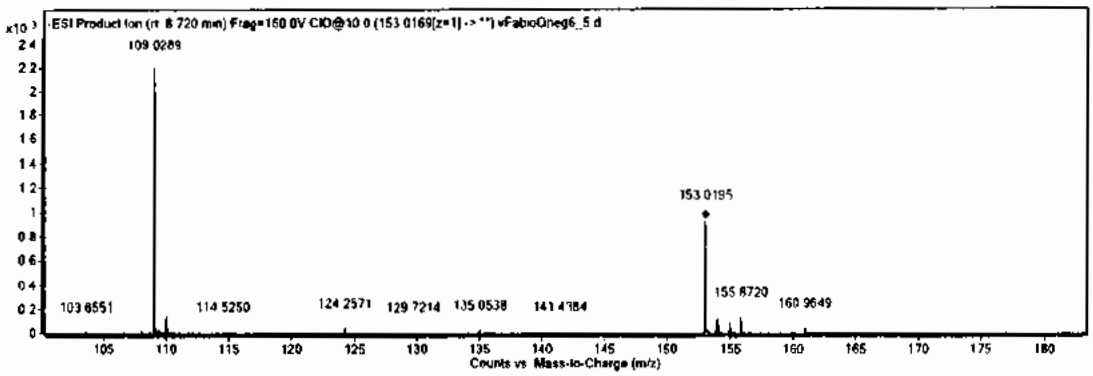


Figure 4.70: MS/MS Spectrum of Protocatechuic Acid

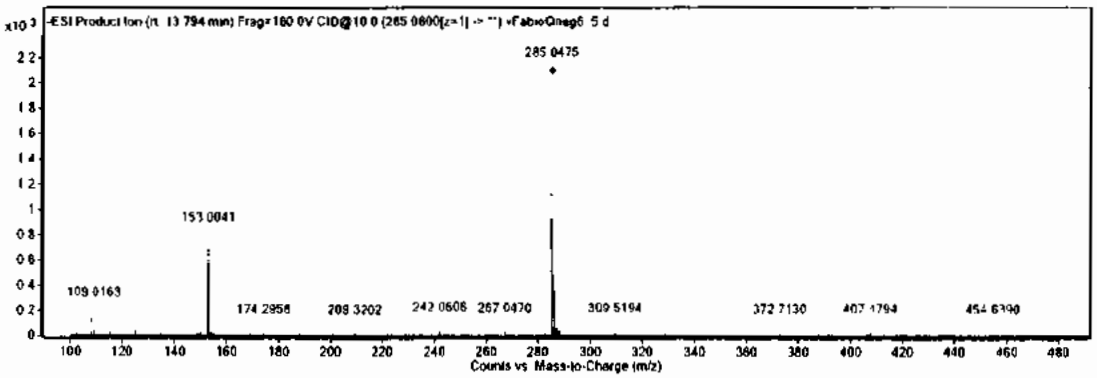


Figure 4.71: MS/MS Spectrum of Kaempferol

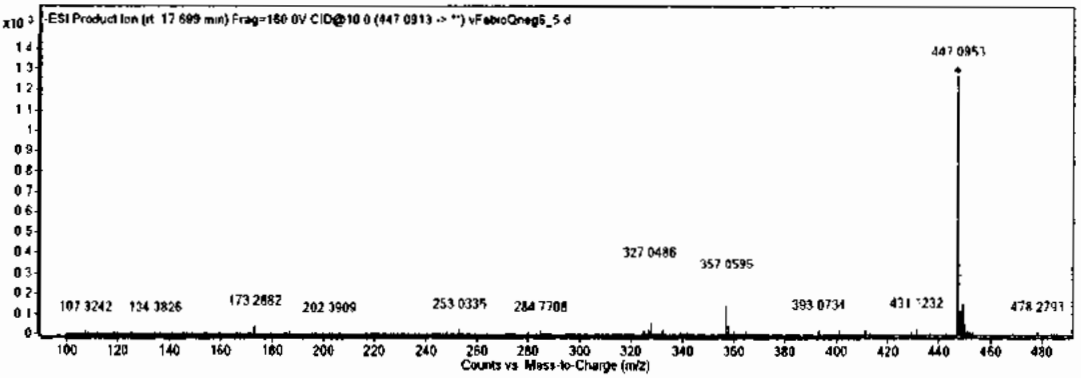


Figure 4.72: MS/MS Spectrum of Quercitrin

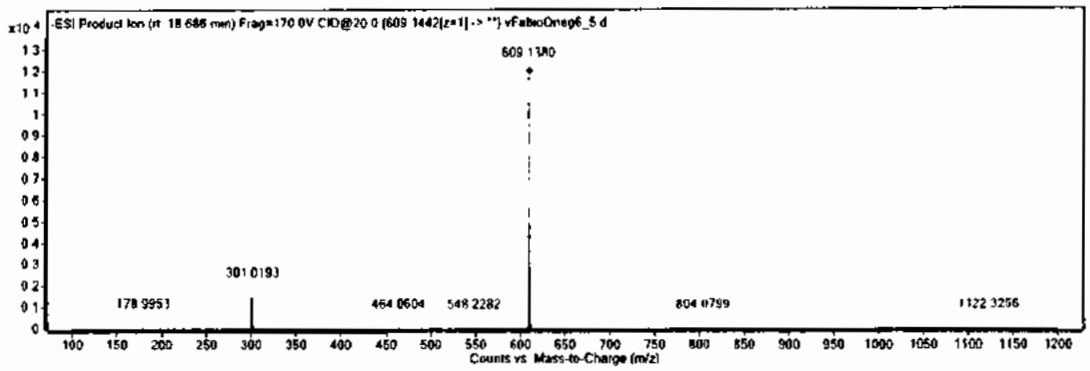


Figure 4.73: MS/MS Spectrum of Rutin

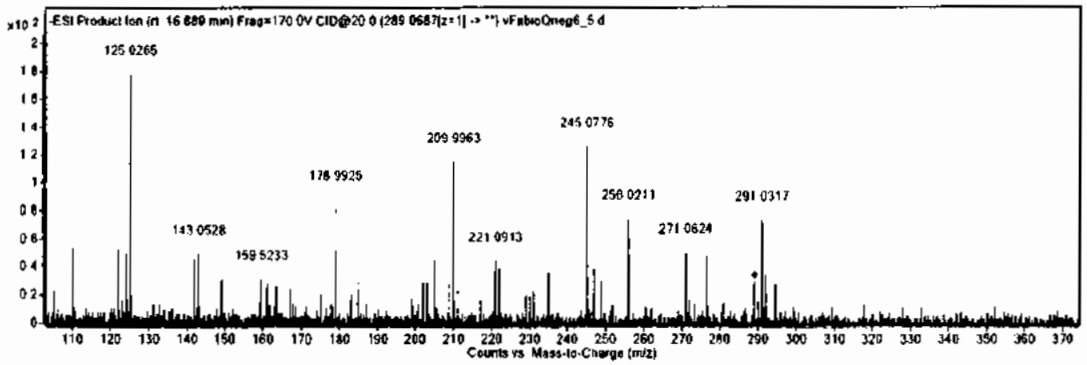


Figure 4.74: MS/MS Spectrum of Epicatechin

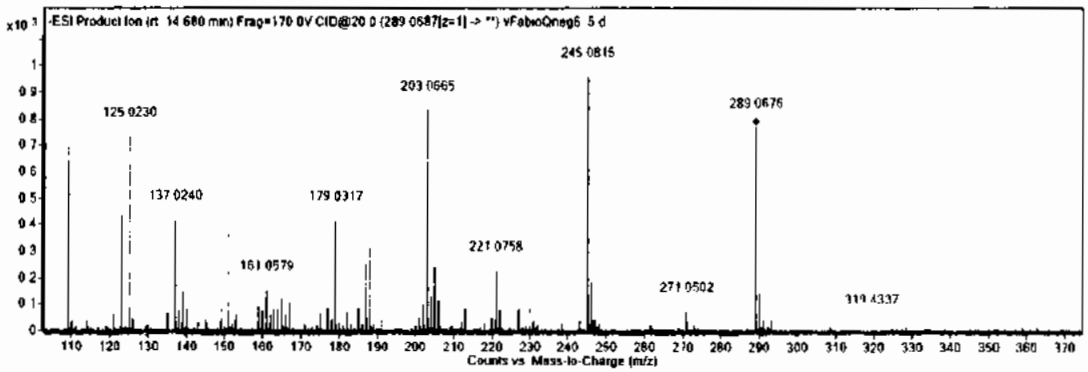


Figure 4.75: MS/MS Spectrum of Catechin

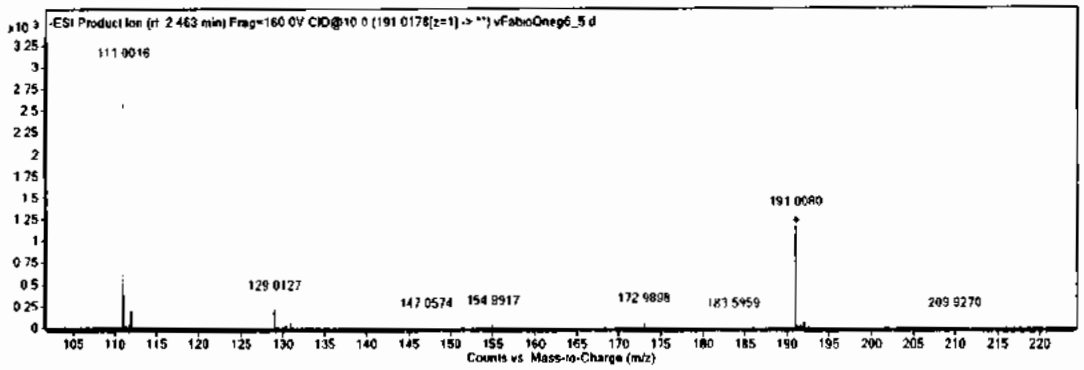


Figure 4.76: MS/MS Spectrum of Quinic Acid

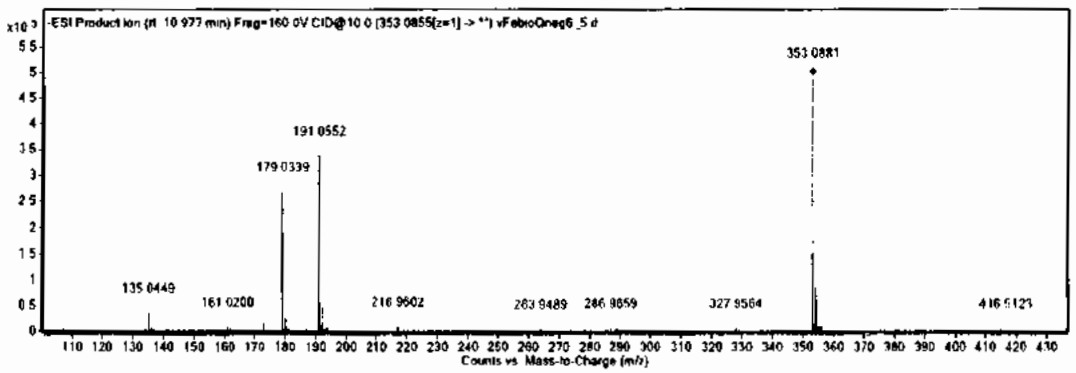


Figure 4.77: MS/MS Spectrum of Chlorogenic Acid

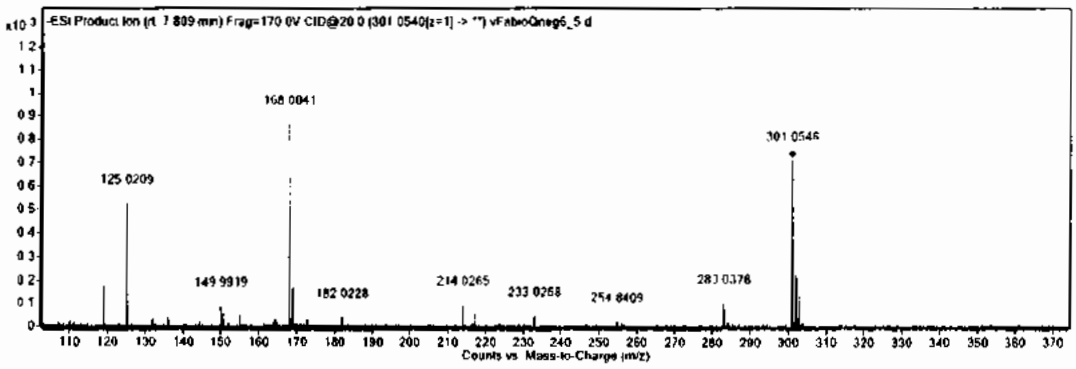


Figure 4.78: MS/MS Spectrum of Quercetin

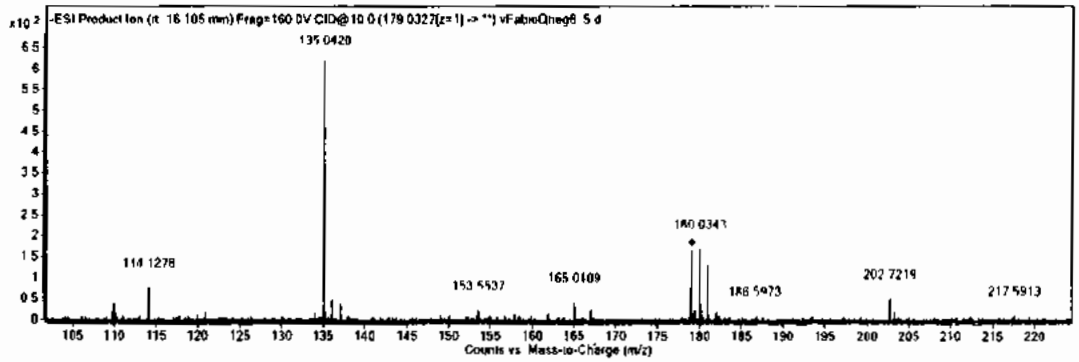


Figure 4.79: MS/MS Spectrum of Caffeic Acid

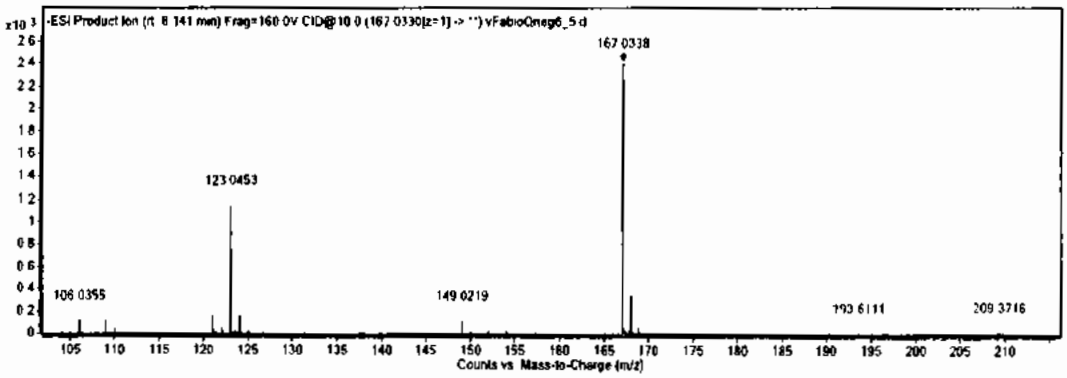


Figure 4.80: MS/MS spectrum of Vanillic Acid

DISCUSSION

5. DISCUSSION

5.1. Discussion

Internal chemical environment of body is regulated through liver by metabolizing the exogenous and endogenous metabolites that are reported to cause the liver damage by oxidative stress (Wang *et al.*, 2016). Liver diseases i.e. fibrosis and cirrhosis are developed by ROS. Liver damage by various hepatotoxins have serious consequences in the body. Despite the remarkable progress in medicines with synthetic drugs for treatment of liver disorders, acute and chronic liver disorders are not often treated satisfactory because they cause serious side effects, not available widely and are not cost effective (Rao *et al.*, 2012).There are many studies that have shown that plant originated medicines play significant role for treatment of liver disorders because they have ingredients possessing strong antioxidant and hepatoprotective nature (Park *et al.*, 2000; Tang *et al.*, 2006; Yue *et al.*, 2011; Gong *et al.*, 2012). Medicinal plants have an important role for drug discovery and primary health care of inhabitants of 80% rely on plants in developing countries. In spite of progress in the field of synthetic medicines, 25% prescribed medicines directly or indirectly are derived from plants in industrialized countries (Newman *et al.*, 2000).

A small proportion of hepatoprotective potentioal of plants have been pharmacologically evaluated for their safety and efficacy (Rao *et al.*, 2012). To contribute for searching the safe liver protecting agents, present study was focused for investigation of hepatoprotective effect of selected medicinal plants collected from various localities of Poonch division (Bagh, Haveli and Poonch) of Azad Jammu and Kashmir, Pakistan. This study was comprised of two parts. Part-I of study was the screening of selected medicinal plants with hepatotoxicity induced on CCl_4 in mice while part-II of this study was comprising about detailed investigation of most active hepatoprotective plants (*G. wallichianum*, *E. parvifolia* and *A. alpinum*) for hepatoprotective effect with different fractions of these three plants through solvent-solvent fractionation, antioxidant activity and phytochemical analysis.

Our selected plants were based on their folkloric belief that these plants are effective to treat different ailments such as diabetes, Jaundice, kidney problems, eye diseases and gastrointestinal disorders. Literature survey revealed that methanolic extract of these selected plants from collected area, have been tested neither for the hepatoprotective effects nor for any other pharmacological activity experimentally.

Extraction is an important step for separation of phytoconstituents (phenolic and flavonoid) contents of plants before any pharmacological activity (Murugan and Parimelazhagan, 2014). In our study, ten selected plants were subjected for crude methanolic extraction. Methanol and ethanol are proved important solvents for extraction and methanol is an important one that is effectively used for extraction of phytoconstituents (Shi *et al.*, 2006). Variuos medicinal plants extracts show highest biological activities (Pandian *et al.*, 2006). Logical pathway is followed to analyze the plant for their pharmacological efficacy and biological safety. Folk medicines of plants origin are tested for their hepatoprotective effects through liver damage in experimental animals (Suja *et al.*, 2002).

Carbon tetra chloride (CCl_4) is widely used hepatotoxic agent for the induction of liver toxicity in experimental animals in the laboratories for pharmacological analysis of natural products and chemically synthesized drugs (Aghel *et al.*, 2007: Ritesh *et al.*, 2015). Plants extract accelerate the liver regeneration and decrease the leakage of serum enzymes into circulation. Silymarin (flavonolignan of *Silybum marianum*) is an important antioxidant with strong free radical scavenger and hepatoprotective agent. It is popular for protecting liver and its recovery process for which it is mainly used as anti-inflammatory, immunomodulatory and hepatocurative. Treatment with Silymarin has proved mild inflammation, less fibrosis and low rate necrosis (Kim *et al.*, 2008). It is useful for the hepatoprotection in hepatobiliary disorders, viral hepatitis, alcoholic liver diseases and liver toxicity by drugs or toxin. It has strong activity for damaged liver regeneration, effective for both acute and chronic hepatitis having safety profile, therapeutic efficacy and better patient tolerability at affordable price. Its LD_{50} is 400 mg/kg in mice and 385mg/kg for rats with variability with infusion rate (Ghosh *et al.*, 2010). During current studies, it has been used as reference SD, as positive control having liver protective effect against CCl_4 hepatotoxicity.

During the current analysis 2000 mg/kg ME dose of each selected plant (VCME, DRME, GWME, EPME, RBME, AAME, ODME, FNME, TSME and PMME) by oral route administered to mice has not shown any toxic effect which confirmed that these ten selected plants are nontoxic and LD_{50} is beyond the 2000 mg/kg of each plant extract. Daily administration of crude methanolic extract (ME) of selected plants extract dose (2000 mg/kg) in mice for 14 days did not cause any of toxicity. In our study all the selected plants extract for study seemed to safe for further study their hepatoprotective effect in mice against CCl_4 induced liver toxicity with low ($1/10^{th}$) and high ($1/5^{th}$) dose of safe level of dose (2000 mg/kg) tested for hepatoprotective effects of plant extract in mice (Kiran *et al.*, 2012; Chander *et al.*, 2014; Reddy *et al.*, 2014).

Carbon tetrachloride (CCl_4) is an important toxicant to induce liver injury for evaluation of effect of plant based or synthetic drugs (Aghel *et al.*, 2007). When CCl_4 is induced in body, the P-450 pathway in the liver converts it into highly reactive molecules (trichloromethyl radicals) which causes increase in ROS, promotion of oxidative stress and lipids peroxidation leading to liver injury. As mitochondria in liver cells play important role in apoptosis but due to disruption caused by oxygen free radicals, mechanism of apoptosis and necrosis is increased. When toxicity is induced, it results ALT, AST, ALP and TB to raise due to leakage into serum indicating the liver cells necrosis as a result of elevated lipids peroxidation due to biotransformation of CCl_4 in liver, causing the membrane lipids disintegration and reduction in the antioxidants level in the body (Yu *et al.*, 2010). It is presumed that multiple time dose administration of CCl_4 is required to induce hepatotoxicity in mice because liver has ability to regenerate when acute toxicity induction is stopped resulting the reverse the toxic effect by regeneration process of liver (Timbrell, 2009). Hepatoprotective activity of selected plants was measured by estimation of ALT, AST, ALP and TB and histopathology by comparing with normal control and standard drug treatment groups (G-I and G-II). Results of our study in animals, orally administered with standard drug and crude methanolic extracts (ME) of selected plants after CCl_4 intoxication by intraperitoneal injection, showed the changes in serum ALT, AST, ALP and total bilirubin (TB) which is shown by the table 4.2 and figures 4.1- 4.4 while percent protection of these biochemical parameter in table 4.3. Our results using CCl_4 toxicity in the mice indicate

significant increase ($P < 0.01$) of liver parameters in CCl_4 toxicant control group (G-II). Value of biochemical parameter elevated on CCl_4 induction that caused hepatic injury that resulted the loss of these enzymes into serum (Preethi *et al.*, 2016). Treatment with SD (G-III) and with low and high dose (200 mg/kg and 400 mg/kg) of crude methanolic extracts of selected plants (VCME, DRME, GWME, EPME, RBME, AAME, ODME, FNME, TSME and PMME) in the treatment groups (G. IV- G.XXIII) significantly ($P < 0.01$) decreased ALT, AST, ALP and TB in the serum of mice respectively that were elevated on CCl_4 toxicity. The effect was marginal with 200 mg/kg and the decrease in level of these liver markers was effectively improved with the high dose (400 mg/kg) treatment of all the selected plants extracts that is also represented by calculation of percentage (%) protection (table 4.3). Higher percentage (%) protection was exhibited by treatment of high dose (400mg/kg) of AAME followed by GWME and EPME (figures 4.1- 4.4, table 4.2 and 4.3). Similar findings were also discussed in another study on plants investigation for their hepatoprotective effects on CCl_4 induced liver injury (Chander *et al.*, 2014). The liver was damaged by the CCl_4 intoxication resulting the cellular necrosis, caused by oxidative stress and reactive oxygen species (ROS) produced pathological changes and resulted the leakage of ALT, AST, ALP and TB into the serum, which increased from their normal level. The decreased level of liver biochemical parameters due to treatment effect of methanolic extract with 200 mg/kg and 400 mg/kg is associated with liver recovery (Alshawsh *et al.*, 2011; Nithianantham *et al.*, 2011). These are key marker enzymes to know the functioning level of the liver, playing an important role for assessment of liver functions. ALT is specific liver marker that is an important parameter for detecting the liver toxicity. ALT is present in parenchyma cells and its increased level in serum confirms the damage of liver due to necrotizing agents like CCl_4 while AST has two isoenzymes one of them is present in cytoplasm (80 %) and other in the mitochondria (20%) of liver cells (Sapakal *et al.*, 2008). ALP and TB are also predominantly present in the cytoplasm. Breakdown of RBC caused excessive production of bilirubin due to liver damages on CCl_4 toxicity. Hepatic obstruction leads to increased level of bilirubin that further implies about liver toxicity due to CCl_4 intoxication (Ahsan *et al.*, 2009; Ahmad *et al.*, 2002; Tsala *et al.*, 2010). When CCl_4 was induced in mice, it caused the damage of liver cells (hepatocytes) and increased level of these

liver markers attributed the damage of structural integrity of liver with cellular necrosis, inflammatory cells infiltration and loss of membranes stability. Due to cytoplasmic location, these enzymes are released into circulatory system after hepatocellular damage, indicating hepatotoxicity development (Afzal *et al.*, 2013). Increasing ALT and AST suggest dysfunction, leakage or acute liver damage (Chand *et al.*, 2011). When treatment was performed with selected plants extracts in mice after CCl_4 intoxication then reduction to leakage of these biochemical parameters to circulations provides the evidence that these plants have potential hepatoprotective effect by healing the damaged cellular structures of hepatic parenchyma and regeneration of hepatocytes with restoration of functional integrity of liver. When CCl_4 was induced, it impaired the bile flow and there was increase in ALP and TB level consequently through the bile. The reduction of increased level of ALP and TB was due to stabilizing the cellular membranes and prevention of bile flow in the liver by the effects of selected plants extract in mice. These plants played their role as scavenger of trichloromethyl radical ($CCl_3^{\cdot}$) generated during CCl_4 metabolism and to reduce oxidative stress caused by lipid peroxidation. Plants extracts (200 mg/kg)by oral administration after CCl_4 intoxication attributed the significant ($P < 0.01$) decrease in ALT, AST, ALP and TB, and this decrease in the value was further improved with some variability among these selected plants in dose dependent response as compare to CCl_4 toxicant control group (G-II) which manifests that all these selected plants possess hepatoprotective activity varying from moderate (VCME > ODME > RBME > PMME > FNME > TSME > DRME) to high (AAME > GWME > EPME) as indicated in figures 4.1- 4.4 in their respective groups. The effect of AAME, GWME and EPME with high dose (400 mg/kg) treatment is comparable to standard drug (SD) treatment (G-III), showing highly hepatoprotective effect against CCl_4 induced toxicity.

In current study hepatoprotective effects of selected medicinal plants is good agreement to the study of Ahsan *et al.*, (2009) in which hepatoprotective activities of some medicinal plants were analysed against CCl_4 toxicity induced in rats and results showed the potent hepatoprotective effect with time and dose dependent manner. Current study is further supported by different literature in which various studies have shown the liver protective

effects of plants on CCl_4 induced liver toxicity in animal model (Ahmad *et al.*, 2002; Ahsan *et al.*, 2009; Krishna *et al.*, 2010; Afzal *et al.*, 2013; Ashoush *et al.*, 2013; Sayed *et al.*, 2014).

The highest reduction of ALT, AST, ALP and TB from their increased level in dose-dependent manners have indicated the improvement in liver healing from its damage effects caused by CCl_4 induction. Dose dependent hepatoprotective effect of plant extracts is also agreed with study performed by Kokhdan *et al.*, (2017) in which low dose and high dose (200mg/kg and 400mg/kg) ethanolic extract of *Stachys pilefira* was applied for investigation of hepatoprotective effects against CCl_4 induced liver toxicity in rats. Results of our studied plants show that highest protection of liver was exhibited by three hepatoprotective plant extract (AAME roots, GWME roots and EPME fruits) in dose dependent manner as compared to other studied plants (table 4.2 & 4.3). Current study of hepatoprotective effect of slected plant against CCl_4 induced toxicity in mice have close agreement with the study performed by Parma *et al.*, (2010) about some plants extracts on paracetamol intoxicated mice. In another study various solvents extract of *V. canescens* whole plant collected from Dir upper KPK Pakistan, was examined by Abdullah *et al.*, (2017) whose results also showed the significant effects against CCl_4 induced liver damage in mice. Related studies were performed by Hsouna *et al.*, (2011); Mistry *et al.*, (2013) and Ponmari *et al.*, (2014) about hepatoprotective effects of plants against CCl_4 induced toxicity. The significant difference among some plants under study to reduce the increased concentration of liver biochemical parameters due to CCl_4 intoxication may be due to difference in phytoconstituents possessed by plant part used and also the occurrence of different phytochemicals in different families of these selected plants to which each plant belongs. Phytoconstituents synthesis in plants is attributed to other geographical/ environmental factors such as soil composition, sunlight, temperature and precipitation also (Liu *et al.*, 2015; Cui *et al.*, 2016). Nonsignificant ($P > 0.01$) effects of some selected plants extract to reduce elevated levels of liver enzymes due to CCl_4 intoxication may be due to similar nature of phytoconstituents possessed by these plants having same antioxidant nature and efficacy. Hussain *et al.*, (2014) also investigated the hepatoprotective effect of *Alcea rosea* crude methanolicextract against paracetamol caused hepatic damage in mice and its results are also supportive for our study about hepatoprotective effect of selected medicinal plants extracts against CCl_4 induced toxicity in

mice. The hepatoprotective effect of our selected plants against CCl_4 induced toxicity in mice is credited to different phytoconstituents (mostly phenolic, flavonoids and tannins) that have regeneration ability of damaged liver cells and neutralization of toxic metabolites generated during Cytochrome-P-450 pathway of CCl_4. The phenolic and flavonoids of these plants have free radicals scavenging activity besides the antioxidant activity possessed by these plants that have reversed the toxic effect of CCl_4 induced damage (Serairi-Beji *et al.*, 2018). High antioxidant and anti-inflammatory activity of these plants attributed the protection of liver cells by regeneration process of damaged cells due to CCl_4 induced stress and lipids peroxidation (Laouar *et al.*, 2017; Attia *et al.*, 2018; Ullah *et al.*, 2018), which is evident from reduced level of liver biochemical parameters in the serum as compared to toxicant control group. The hepatoprotective effect of selected medicinal plants in our study is further supported by the liver histopathological analysis. There is normal cellular architecture having intact nuclei, regular shape with normal central vein and portal vein (figure 4.5). There were histopathological changes like necrosis of hepatocytes, fatty changes and sinusoidal dilation, inflammatory cells infiltration, fatty degeneration, bleeding area in the hepatic lobes with the loss of cellular architecture due to CCl_4 intoxication (figure 4.6). It resembles to the results obtained by other studies performed (Hsouna *et al.*, 2011; Kale *et al.*, 2012; Attia *et al.*, 2018; Ullah *et al.*, 2018) on plants against CCl_4 induced toxicity in mice. Treatment with low and high dose (200 mg/kg and 400 mg/kg) of crude methanolic extract of ten selected plants (VCME, DRME, GWME EPME, RBME, AAME, ODME, FNME, TSME and PMME) respectively in the treatment groups (G-IV to G-XXIII) have shown variable recovery ability in protecting the liver cells from injury caused by CCl_4 intoxication (4.8a-4.17a and 4.8b-4.17b). Recovery of histological pattern is seen in the mice treated with low dose (200 mg/kg) as represented in figures 4.8-4.17 (a) with further improvement when treated with high dose (400 mg/kg) of selected plants extracts having optimum results (figures 4.8b-4.17b) comparable to SD (Silymarin100 mg/kg) treatment (G-III) as indicated in figure 4.7 and normal control mice (G-I) shown in figure 4.5 as compared to CCl_4 toxicant control group (G-II) as shown in figure 4.6 (Abou-Ela *et al.*, 2005). Our findings are better agreeable with findings of Grace-Lynn *et al.*, (2012) for studies about Lantana plant against acetaminophen-intoxicated mice. There was normal cellular structure and regeneration of hepatocytes after

treatment with plants extracts (Hsouna *et al.*, 2011; Yang *et al.*, 2011; Laouar *et al.*, 2017; Attia *et al.*, 2018; Ullah *et al.*, 2018) which attributed to significant hepatoprotective potential of selected plants under study. These findings help to decide that hepatoprotective effect of these plants is dose and time dependent (Ahsan *et al.*, 2009). Results reported by another study about some plants extracts provide the evidence of hepatoprotective role of medicinal plants against CCl_4 induced toxicity in mice (Gumma *et al.*, 2017). The possible hepatoprotective effects of these plants extracts is associated with inhibition of lipids peroxidation, free radicals scavenging effect and antioxidant activity (Yam *et al.*, 2007). The process of healing caused the reduction of leakage of biochemical parameters into serum that is strong evidence that selected plants are hepatoprotective. The order of hepatoprotective effect of these selected plants extract is AAME > GWME > EPME > VCME > ODME > RBME > PMME > FNME > TSME > DRME in dose dependent manner. It may be due to mechanism of inhibition of peroxidation and improvement in antioxidant level (Yang *et al.*, 2017). Three plants (*A. alpinum* roots, *G. wallichianum*roots and *E. parvifolia* fruits) showed the highest hepatoprotective effects attributed with highly significant reduction of increased level of liver markers as compared to TC (CCl_4) control group (Mekky *et al.*, 2016). Treatment results of these three plants are comparable to SD treatment (Silymarin 100 mg/kg) and normal control group of animals (G-I). The highest protection by these three plants to liver is strongly verified by histopathological observations where the less necrosis of hepatocytes, mild degree of degeneration, less inflammatory cell, well-formed hepatocytes separated by sinusoids were seen due to 400 mg/kg dose effect of these plants extracts (4.13b, 4.10b and 4.11b) respectively. Three selected plants (*G. wallichianum*, *E. parvifolia* and *A. alpinum*) having better protective effects against CCl_4 induced toxicity in mice in our study were shortlisted for further study to know the antioxidant activity and phytoconstituents nature that may have caused hepatoprotective effects in mice.

Pharmacological investigation of medicinal plants in most cases is limited to crude extracts and hytochemicals separation from the mixture is ignored due to which expected biological activities are not fully known. Herbal formulation could be efficiently approached when mixture of phytochemicals is separated by solvent-solvent partitioned methods. Polar solvents separate polar compounds while less polar phytoconstituents are separated through

less polar solvents (Zakaria *et al.*, 2016). Extraction yield of phytochemical compounds increases with increasing polarity of extraction solvent (Almey *et al.*, 2010). In our experiment, crude methanolic extract of most active plants (*G. wallichianum*, *E. parvifolia* and *A. alpinum*), produced the different quantity of each fraction of respective plant with solvents-solvent partition. Results (table 4.4) indicate that there was highest quantity of aqueous fraction ofGWAF (112g) followed by GWEF (31.4g), GWBF (21.3g), GWHF (14.6g) and GWCF (11.5g). The fractionation of crude methanolic extract of EPME produced highest quantity in an EPAF (98.62g) followed by EPEF (26g), EPCF (14.45g), EPBF (10.2g) and EPHF (9.8g). Fractionation of crude methanolic extract of *A. alpinum* (AAME) produced the different quantity of each fraction in which highest quantity was in AAAF (106 g) followed by AAEF (29g), EPBF (16g), AACF (12.68g) and AAHF (11.7g). Highest quantity of fraction is possessed by AF followed by EF and BF as shown in table 4.4. It shows that quantity of more polar compounds higher than less polar compounds is possessed by the *G. wallichianum*, *E. parvifolia*and *A. alpinum*. When the systematic separation of secondary metabolites is applied then the herbal formulation to treat diseases becomes more efficient. Partition of crude extract of *Euphorbia wallichi* by solvents like ethyl acetate, chloroform, n-hexane, water and n-butanol into fractions exhibited excellent biological activity (Ihsan-Ul-Haq *et al.*, 2012). The separation of mixture into its components enhances efficacy and specificity for standardization and characterization of plants based drugs. Efficacy of plants derived drugs for stabilizing cellular membranes and free scavenging effect to free radicals in biological systems is increased by partition of mixtures from extracts (Jalil *et al.*, 2015). In our present study different solvents fractions of most active hepatoprotective plants (*G. wallichianum* roots, *E. parvifolia* fruits and *A. alpinum* roots) have been tested to determine the most active fractions of these plants for hepatoprotective effect against CCl_4 induced liver damage in mice. Results showed that ALT, AST, ALP and TB levels were increased with significant effects ($P < 0.01$) in toxicant control group (G-II) due to CCl_4 intoxication by intraperitoneal injection (Serairi-Beji, 2018). Our results (figures 4.18-4.21) show that SD (100mg/kg) treatment and fractions of *G. wallichianum* (GWHF, GWCF, GWEF, GWBF and GWAF) with low dose (200 mg/kg) after CCl_4 intoxication, significantly ($P < 0.01$) decreased the value of liver biochemical markers respectively. The values of liver

biochemical markers further improved in decreasing with 400mg/kg dose of increased polarity fractions (GWEF, GWBF and GWAF). Highest protective effect was exhibited by GWAF followed by GWEF and GWBF with high dose (400 mg/kg) treatment of these fractions against CCl_4 toxicity as compare to CCl_4 toxicant control group that is comparable to normal control group (G-I) and SD (100mg/kg) treated group (G-III). Treatment effect of GWHF and GWCF with low and high doses (200 mg/kg and 400mg/kg) caused same reducing effects to the elevated level of ALT, AST, ALP and TB as compared to other fractions (figures 4.18-4.21), and it may be due to same nature of none/less polar compounds having similar efficacy possessed by them. The level of liver markers increased from normal control mice (G-I), due to CCl_4 intoxication (Preethi *et al.*, 2016).

Studies about treatment with different fractions of *E. parvifolia* (EPHF, EPCF, EPEF, EPBF and EPAF), with 200mg/kg and 400mg/kg significantly ($P < 0.01$) reduced liver serum markers i.e ALT, AST, ALP and TB as compare to CCl_4 toxicant control group. Highest reduction was exhibited by EPAF followed by EPEF with the high dose (400mg/kg) treatment which is comparable to SD (100 mg/kg) treatment after CCl_4 intoxication as compare to toxicant control group (G-II). The reduction of increased level of ALT, AST, ALP and TB, with 200mg/kg and 400mg/kg dose of EPHF and EPCF was same (with no significant difference between them). EPBF with 200mg/kg and 400mg/kg treatment caused significant ($P < 0.01$) reduction for the elevated levels of above mentioned liver markers but with less effect as compare to other intermediate polar fraction (EPEF) and more polar fraction (EPAF), and higher than less polar fractions (EPHF and EPCF) treatment (figure 4.30- 4.33). This might be due to extraction of intermediate active phytoconstituents during solvent-solvent fractionation. Liver biochemical parameters (ALT, AST, ALP and TB) were significantly reduced with the treatment of 200mg/kg of AAAF followed by AAEF and AABF as compare to CCl_4 toxicant control group, which was further improved to reduce with high dose (400 mg/kg) treatment whose effect is like normal control and SD (100 mg/kg) treatment group (GIII) respectively. There was same non-significant ($P > 0.01$) difference of liver markers with treatment of 200mg/kg and 400mg/kg of AAHF and AACF respectively (figures 4.39 - 4.42). This showed that these none/ less polar fractions might have same nature of non-polar phytoconstituents having less hepatoprotective effect. It is further supported by

liver histopathological studies of mice. Degeneration of hepatocyte, loss of cells architectures, ballooning, focal necrosis, dilated sinusoids and inflammatory cells infiltration show that liver injuries were caused by CCl_4 intoxication (figure 4.23). Damage in sinusoids and disruption in hepatocytes is seen as compare to normal control mice (figure 4.22) and standard drug treatment (figure 4.24). There is less degeneration and necrosis when treatment with low dose of extract is performed and this recovery is mild in groups treated with HF and CF of three extracts as shown in figures 4.25 – 4.26 (a, b), 4.34 - 4.35 (a, b) and 4.43 – 4.44 (a, b) respectively (Yang *et al.*, 2017). Similarly, highest regeneration is observed with well-recovered hepatocyte, separated by sinusoids spaces in groups treated with EF, BF and AF of these three plants (Ullah *et al.*, 2018; Abdelhafez *et al.*, 2018), as indicated in figures 4.27 – 4.29 (a, b), 4.36 - 4.38 (a, b) and 4.45 - 4.47(a, b) respectively, as compared to NC mice (figure 4.22) and SD (100 mg/kg) treated group (figure 4.24) of mice. Highest protective effect of *G. wallichianum* fractions (GWEF, GWBF and GWAF), *E. parvifolia* fractions (EPEF and EPAF) and *A. alpinum* fractions (AAEF, AABF and AAAF) was attributed to reduce the elevated level of liver markers that were increased on CCl_4 intoxication. Highest activities of these fractions after CCl_4 intoxication is due to polar nature of phytochemicals that were partitioned by these solvents during fractionation. The decrease in elevated levels of liver biochemical parameters shows that these fractions have effectively detoxified the metabolites that were generated due to CCl_4, produced ROS and lipids peroxidation (Yang *et al.*, 2017; Abdelhafez *et al.*, 2018). Most hepatoprotective effect was indicated by reducing the increased level of liver biochemical parameters by more cellular regeneration, less necrotic effect, less dilated sinusoids and less inflammatory cell infiltration mechanisms. In present results, intermediate polar fraction (EF) and more polar fractions (BF and AF) of extract have highest hepatoprorotective role after CCl_4 intoxication which it attributed to intermediate and more polar compounds mixture in these fractions. These compounds may be flavonoids, tannins, saponnins and polyphenolic distributed in these fractions (Almey *et al.*, 2010). Results of our study showed that most hepatoprotective activity was possessed by the fractions of intermediate polar and polar solvents (EF and AF). The aqueous fractions followed by ethyl acetate fractions and butanol fractions in case of current studied plants, have shown highest hepatoprotective effect against CCl_4 induced toxicity in mice. The less

polar fractions (HF and CF) of these three studied plants with treatment of low and high doses (200 mg/kg and 400 mg/kg) have shown less hepatoprotective effect, attributed to less reducing effect on elevated level of liver marker enzymes (ALT, AST, ALP and TB) as indicated in figures 4.18 - 4.21, 4.30 - 4.33 and 4.39 - 4.42, and less cellular regeneration, less improvement in cellular necrosis and more dilated sinusoids as well as infiltration of fats with treatment of 200mg/kg and 400mg/kg as compare to other studied fractions of these plants (figures 4.25 - 4.26 (a, b), 4.34 - 4.35 (a, b) and 4.43 - 4.44 (a, b) respectively). This might be due to inhibitory effects of some phytoconstituents in separate form or separation of less polar compounds of same nature in n-hexane and chloroform during solvent-solvent partition of crude methanolic extract of these three short listed most active plants. Less hepatoprotective effect may be due to low antioxidant nature and less anti-inflammatory activity of the phytoconstituents present in these fractions (HF and CF). When these ingredients were in crude mixture of methanolic extract, then more activity might be due to synergistic or combined action of both non-polar and polar compounds (Chen *et al.*, 2014; Nguyen *et al.*, 2015). The less reduction ability of EPBF as compare to its other polar fractions (EPEF and EPAF) might be due to separation rate or less contents of active phytoconstituents in this solvent (n-butanol) during fractionation. Leakage of these enzymes into serum is elevated when damage is caused by the CCl_4 intoxication attributed with oxidative stress production due to free radicals generated by biotransformation of CCl_4 to trichloromethyl radical, membranes damage through lipid peroxidation and intrahepatic biliary obstruction. Our results about hepatoprotective effect of different fractions of methanolic extract of short listed active plants against CCl_4 induced toxicity in mice are supported by studies about *Alchornea cordifolia* againstCCl_4 intoxicant micein which ethyl acetate fraction was found most active to reduce the elevated of liver markers (Osadebe *et al.*, 2012). Hepatoprotective activities of *Juniperus phoenice* extracts against CCl_4 induced toxicity was also studied which showed the potent hepatoprotective effect (Laouar *et al.*, 2017). Hepatoprotective effect of ethyl acetate fraction, petroleum ether fraction and water fraction of *Bauhina purpurea* leaves on paracetamol intoxicated mice in previous study showed that ethyl acetate fraction (EF) followed by aqueous fraction (AF), are most active to reduce increased level of liver enzymes (Zakaria *et al.*, 2016), which further supports us

to conclude that active fractions of *G. wallichianum* roots extract are;GWAF followed by GWEF and GWBF, while *E. parvifolia* fruits extract have; EPAF and EPEF to some what EPBF and *A. alpinum* root extract have AAAF followed by AAEF and AABF.The most active fractions in these three plants under study are AF and EF because they have highest significant effect to reduce liver biochemical parameters, attributed the regeneration of liver parenchyma and hepatocytes with strong antioxidant activity and scavenging effect to free radicals, produced due to CCl_4 intoxication, comparable to standard drug control group and normal control group respectively as compare to TC (CCl_4) group. The protective effect of these fractions (EF, BF and AF) was dose dependent also. As the concentration was increased, the hepatoprotective effect was also increased; attributed to more improvement for reducing the increased level of liver biochemical parameters as compare to toxicant control group, and more hepatocellular recovery of damaged cells, no necrosis, mild degree of degeneration and normal central vein (Agbafor *at al.*, 2014; Talluri *et al.*, 2018). Necrotizing agents like CCl_4 has caused sufficient liver injury to release excess of bilirubin in the serum (Nithianantham*et al.*, 2011). The more effect of EF, AF and BF to protect from CCl_4 induced liver damage is due to intermediate polar and polar compounds separated during fractionation. The phenolic and flavonoids are polar and effective phytoconstituents that play important role for detoxification of toxins that is closely agreed with studies of Serairi-Beji et *al.*, (2018). The polyphenols have highest antioxidant activity and free radicals scavenging effect against toxins produced in the body. HF and CF fractions have caused less protective effect as compare to other fractions due to different nature of compounds that may have less ability to regenerate the damaged cells of liver, attributed the less antioxidant activity and low scavenging effect to free radicals produced during oxidative stress and lipids peroxidation due to CCl_4 intoxication. Reactive oxygen species (ROS) are most frequent pro-oxidants which are generated by normal metabolism or different toxins induced toxicity. The harmful effects are mitigated by useful antioxidants intake. The natural antioxidants obtained of plant have no adverse side effects in the body against the free radicals by scavenging activity as compare to synthetic antioxidants that induce serious side effects in the body (Oyinloye*et al.*, 2016). Antioxidant nature of plants is due to phytochemicals possessed by those medicinal plants. Plants rich in phenolic and flavonoids are highly antioxidant in nature

(Laouar *et al.*, 2017; Ullah *et al.*, 2018). The CCl_4 intoxication causes the adverse effects in body such as liver damage due to result of free radical generation, which cause oxidative stress and lipids peroxidation. The imbalance between ROS and antioxidant production impairs the hepatic disorder (Galicia-Moreno *et al.*, 2014).

To investigate radical scavenging effect of medicinal plants extracts, DPPH assay is related to inhibition of free radicals to estimate the antioxidant activities (Chand *et al.*, 2012). DPPH free radical has maximum absorbance at 517nm which results in noticeable discoloration to yellow from deep violet on acceptance of an electron or hydrogen atom (H^+) from donating species (antioxidant) and results the decrease in absorbance (Mosquera *et al.*, 2007). Hepatoprotective activity of herbal products is associated with their antioxidant activity and free radicals scavenging effects. For better understanding the biological activities like the hepatoprotective effect of plants extracts; antioxidant activity is efficient and reliable assay (Hasan *et al.*, 2009). DPPH is decolorized by an antioxidant and radical scavenging action is directly and reliably determined by this assay (Magama *et al.*, 2013). The IC_{50} value is calculated to determine the antioxidant capacity of an extract, which is inversely related to antioxidant activity. It is the concentration of antioxidant (extract) that inhibits the 50% of DPPH radical (Moyo *et al.*, 2013). DPPH assay provides basic information about scavenging effect of extract free radicals. In our study, scavenging effect (%) to free radical (DPPH) was increased with concentration of antioxidant sample. Present study about antioxidant activity of metanolic extracts and fractions of three most hepatoprotective active plants (*G. wallichianum*, *E. parvifolia* and *A. alpinum*), the results show that concentration dependent potential against DPPH was exhibited by samples and the reference standard i.e. ascorbic acid (AA). The IC_{50} calculation was performed by regression equation of ascorbic acid to know antioxidant activity (figure 4.51). In our study about *G. wallichianum*, the GWME and fractions (GWHF, GWCF, GWEF, GWBF and GWAF) exhibited moderate to high rate of DPPH scavenging (%) activity and the free radical scavenging effect was increased with increase in concentration of extract/fraction. Highest scavenging effect (%) was performed by GWEF (92.3±0.6%) at 100µg/mL, comparable to standard free radical scavenger i.e. ascorbic acid (95.3±1.2%) as indicated in figure 4.48. The IC_{50} value of crude methanolic extract and its fractions showed moderate to high antioxidant activity. The IC_{50} in our results

shows that GWAF has lowest IC_{50} value (29.92µg/mL) which is less than IC_{50} value of standard antioxidant ascorbic acid (31.90µg/mL). This shows that polar fraction (GWAF) and intermediate polar fraction (GWEF), have exhibited highest antioxidant activity followed by GWBF and GWME, as indicated in table 4.7. Similarly, our study about *E. parvifolia* crude methanolic extract (EPME) and its fractions (EPHF, EPCF, EPEF, EPBF and EPAF) have exhibited moderate to high rate of DPPH scavenging (%) activity. Free radical scavenging effect was increased with increasing concentration of extract/fraction as indicated in figure 4.49. Highest scavenging effect (%) was performed by EPEF (92.3±0.3%) at 100µg/mL, comparable to standard free radical scavenger ascorbic acid (95.3±1.2%). The IC_{50} of crude ME and its fractions showed variability from moderate to high antioxidant activity. The IC_{50} in our results shows that EPAF has lowest IC_{50} value (32.07µg/mL) which is comparable to IC_{50} value of standard antioxidant ascorbic acid (31.90µg/mL), as indicated in table 4.8. This showed that polar fraction (EPAF) has exhibited highest antioxidant activity. In current study about crude ME of *A. alpinum* roots (AAME) and its fractions (AAHF, AACF, AAEF, AABF and AAAF) showed potential free radical scavenging (%) effect against DDPH radical which indicates the increasing scavenging effect with increasing concentration (Shehab *et al.*, 2015). Highest scavenging effect (%) is exhibited by AAEF (96.4±0.6) at 100µg/mL followed by AAME, AABF, AAAF, AACF and AAHF, as compare to scavenging (%) effect of standard ascorbic acid (95.3±1.2%) to DPPH (figure 4.50). AAAF showed lowest IC_{50} value (25.51µg/mL) exhibiting highest antioxidant activity. Similarly, the promising high antioxidant activity is shown by AAEF (IC_{50} 30.06µg/mL) which is also comparable to the IC_{50} value of standard antioxidant ascorbic acid (IC_{50} 31.90µg/mL) as shown in table 4.9.

The high activity is due to nature of phytoconstituents seperated in aqueous and ethyl acetate fractions. The free radical scavenging activity of plants extract depends on the nature of solvents used for separation of phytoconstituents (Ullah *et al.*, 2018). None polar solvent extracts have shown less antioxidant activity. More antioxidant activity is attributed to polar solvents extract/fraction. Study shows that free radical scavenging effect of 12 medicinal plants in which seven plants showed more than 70% scavenging effect (Aqil *et al.*, 2006). DPPH radical scavenging activity of whole plant extract of *G. wallichianum* from Northern

areas, Pakistan, was previously studied by Ismail *et al.*, (2009) in which the ethyl acetate extract showed highest antioxidant activity with lowest IC_{50} value (19.05±0.90), followed by n-butanol extract (IC_{50} 24.133±0.56 µg/mL) and aqueous extract (IC_{50} 25.35±1.20 µg/mL), which is higher than our results exceptionally, which may be due to geographical distribution difference of this plant and part used for the study (Laouar *et al.*, 2017). The radical scavenging effect (%) of all these three plants (*G. wallichianum*, *E. parvifolia* and *A. alpinum*) extracts/fractions increased with increasing concentration (10-100 µg/mL) of each extract during the assay, as indicated in figures 4.48 - 4.50. Similarly, the extract whose IC_{50} is low that is strong antioxidant in all the three active plants i.e *G. wallichianum*, *E. parvifolia* and *A. alpinum* (tables 4.7-4.9). AAAF and AAEF have more pronounced antioxidant activity than standard AA. The phytochemicals present in intermediate polar fractions (EF) and more polar fractions (BF and AF) are mixture of polyphenolic and flavonoids that may have strong antioxidant activity that is supported by studies performed by Ullah *et al.*, (2018). The crude extracts have high antioxidant activity as compared to other nonpolar fractions because in the mixture the phytochemicals have synergistic effect for scavenging the free radical DPPH (Jumuna *et al.*, 2012). Studies have shown that different species of *Ramie* leaves play important antioxidant role for health benefits having percent scavenging effect (%) increased with increase in concentration and higher antioxidant activity with low EC_{50} value (Chen *et al.*, 2014). The concentration based scavenging effect to free radical of plant extract occurs up to 100 µg/mL (Preethi *et al.*, 2016). DPPH scavenging activity is increased in dose dependent manner as depicted from various studies in which activity is increased at concentration range of 60µg/mL to 180µg/mL (Do *et al.*, 2013). This supports to our study where the scavenging effect (%) of all extract to DPPH is correlated to concentration of samples. As the IC_{50} is inversely related to antioxidant capacity. The IC_{50} value of extract in solvent increases with increasing water contents in that solvent (Murugan and Parimelazhagan, 2014). Another study shows that main antioxidant compounds are phenolic and flavonoids and there is correlation between antioxidant activity and phenolic contents (Liu *et al.*, 2009). Strong antioxidant activity of EF of current study is also ssupported clearly by another study performed by Osadebe *et al.*, (2012) on *Alchorne cardifolia* in which ethyl acetate fraction of its methanolic extract was used against DDPH analysis. Another study has

demonstrated that low IC_{50} value of extracts have strong antioxidant activity of ethyl acetate and aqueous extract, as compared to other extracts (hexane, chloroform and ethanolic), which also supports our investigation of antioxidant activity for DPPH assay of three plants extracts (Devika *et al.*, 2016). In an earlier investigation of antioxidant activity by DPPH assay of another plant of same family by Nazir *et al.*, (2018) from KPK Pakistan with methanolic extract showed the high antioxidant activity of fruit extracts of *E. Umbellata* with chloroform fraction (IC_{50} 40µg/mL), ethyl acetate fraction (IC_{50} 45 µg/mL) and butanol fraction (IC_{50} 60µg/mL) against standard ascorbic acid (IC_{50}30µg/mL). Antioxidant activity of plants are associated to phenolic and flavonoids that are abundant plants metabolites (Dia and Mumper, 2010). Studies have shown that medicinal plants have potent antioxidant activity due to natural antioxidants (Gomes *et al.*, 2003; Couladis *et al.*, 2003). Extracts have proved potent antioxidant activity due to nature of phytochemical. Antioxidant activity of phytochemicals plays an important role of liver protection in mice against hepatotoxicity induced. When aqueous extract of *Ziziphus jujuba* leaves was applied for liver healing against paracetamol induced hepatic injury then it came into observation that *Z. jujuba* leaves have potent hepatoprotective effect because of antioxidant activity (Preethi *et al.*, 2016). Plants extracts or herbal formulation protect the liver injury and tend to normalize the toxicity due to presence of phytochemicals (such as triterpenes, flavonoids and phenolic compounds) having radical scavenging effect and antioxidant activity (Rajesh and Latha, 2004; Laszczyk *et al.*, 2006). Hepatoprotective effect of plant extracts/fractions is ascribable to antioxidant activities and scavenging effect to free radicals (Yam *et al.*, 2007). The fractions (EF, BF and AF) of our short listed active plants which have shown highest antioxidant activity are more hepatoprotective as compared to other fractions (HF and CF).

Hepatoprotective effect of plants extract is due to phytochemicals contents especially the phenolic and flavonoids. Variety of biological activities i.e. anti-inflammatory, hepatoprotective and antioxidant activity have been reported about phenolic compounds (Amarowiez, 2007; Hadriche *et al.*, 2016). Literature has no documented record for TPC in our plants extracts. Methanol extract is good for extraction of phytoconstituents like phenolic and flavonoid contents (Murugan and Parimelazhagan, 2014). Total phenolic contents in our study were calculated by regression equation of gallic acid (GA) calibration curve (figure

4.52). It is proved from the results obtained in the current analysis of TPC in ME of *G. wallichianum* and its active fractions, that GWME has highest TPC (237.094±0.695µg/mg GAE) followed by GWEF (183.387±0.965µg/mg GAE), GWAF (154.640±2.104µg/mg GAE) and GWBF (105.579±1.645µg/mg GAE) respectively (table 4.10).

The results show that *E. parvifolia* has highest TPC in EPME (242.581±0.708µg/mg GAE) followed by EPAF (142.430±1.645µg/mg GAE), EPEF (125.584±1.750µg/mg GAE) and EPBF (68.322±1.520µg/mg GAE). *A. alpinum* has the highest TPC in AAME (192.892±0.814µg/mg GAE) followed by AAEF (185.164±0.836µg/mg GAE), AAAF (165.304±0.708µg/mg GAE) and AABF (109.433±1.771µg/mg GAE) respectively (table 4.10). Results of *G. wallichianum, E. parvifolia and A. alpinum* has high TPC in their crude methanolic extract (GWME, EPME and AAME) because there is combined mixture of all the phenolic present in each plant. The TPC in EF is high followed by AF and BF of GW and AA. There are agreeable findings in our results about TPC in these plants with methanolic extract and highly active fractions (ethyl acetate fraction, n-butanol fractions and aqueous fractions) with previous study of another plant extract (Murugan and Parimelazhagan, 2014). Here our results showed more TPC in plants under study than another study performed for *Juniperus pheonicea* barries with an aqueous extract and methanolic extract (Laouar *et al.*, (2017). Ihsan-ul-Haq *et al.*, (2012) also studied TPC in *Euphorbia willichii* applying the same procedure and determined the % contents of phenolic compounds showing that fractionation process separates these contents efficiently. There is documented relationship between antioxidant activity and TPC (Shehab *et al.*, 2015). It is confirmed that antioxidant nature of TPC exhibits the strong hepatoprotective effects (Laouar *et al.*, 2017). It is also anticipated that increase in level of TPC in solvent extract exhibit increasing antioxidant activity (Bolling *et al.*, 2010) which supports our findings in EPAF, EPEF and EPBF but there are also findings which show the low level of TPC exhibit the high antioxidant activity (Peschel *et al.*, 2007) which supports our findings in crude extracts, aqueous fractions (AF) and ethyl acetate fractions (EF) of *G.wallichianum* and *A. alpinum* with the exception of GWBF and AABF in which the effect is concentration dependent. Low antioxidant activity exhibited by high level of TPC is also considered to relate with poor specificity of TPC in assay, having number of phenolic groups react to Folin-ciocalteu reagent differently and antagonistic way

(Singleton *et al.*, 1999; Escarpa *et al.*, 2001). Other factor regarding the variations in separation of polyphenolics is polarity of solvents extracts in which ethyl acetate is intermediate polar and water is more polar than other solvent used in our study. Phenolic compounds are very important phytochemicals that have one or more hydroxyl group's attachment with more aromatic rings, which impairs characteristics effects for biological activities. Anyhow, there may be significant difference of phytochemical in different extracts of plants with different parts used during phytochemical analysis (Chen *et al.*, 2014). Hepatoprotective effect and antioxidant activity are correlated to TPC (Yen *et al.*,1993; Yang *et al.*, 2002; Yoshikawa*et al.*, 2002). Important plants polyphenolics include phenolic acid, tannins and flavonoids in which flavonoids are most important polyphenols. The diversity in the structures of these compounds is attributable to oxidative linkage formation and flavonoids exhibit important biological activities due to this property (Zakaria *et al.*, 2016). In our study, the total flavonoids contents (TFC) were calculated by regression equation of quercetin calibration curve (figure 4.53). It is apparent from the results (table 4.11) that. *G. wallichianum* has highest total flavonoids contents (147.035±0.404 µg/mg QE) in GWAF, followed by GWEF total flavonoids contents (118.171±0.194 µg/mg QE), GWME total flavonoids contents (95.843±0.582 µg/mg QE) and GWBF total flavonoids contents (84.129±0.112 µg/mg QE) respectively. In the case of *E. parvifolia*; EPME have the highest TFC (196.027±0.194 µg/mg QE) followed by EPEF (113.705±0.388 µg/mg QE), EPAF (99.532±0.336 µg/mg QE) and EPBF (45.233±0.624 µg/mg QE) respectively (table 4.11). Similarly, AAAF has the highest TFC (150.659±0.489 µg/mgQE), followed by AAEF (132.279±0.297 µg/mgQE), AAME (128.267±0.514 µg/mg QE) and AABF (95.778±0.404 µg/mg QE) respectively (table 4.11). More polar fractions (AF) contain the highest total flavonoids in each plant (*G. wallichianum* and *A. alpinum*) followed by EF, methanolic extracts and BF of GW and AA. There is variability in TFC of *E. parvifolia* i.e highest TFC in its crude methanolic extract but least quantity of TFC (45.233±0.624 µg/mg QE) in n-butanol fraction of *E. parvifolia*. The results (table 4.11) indicate that intermediate polar fraction (EPEF) contains more TFC than its more polar fraction (EPAF). Phytoconstituents; phenolic and flavonoids exhibit different biological activities (anti- carcinogenic, anti-inflammatory and hepatoprotective effects) which are associated with antioxidant activities

(Briskin, 2000; Ruth, 2004; Afran and Kader., 2006; Devika *et al.*, 2016). Phytochemical composition (especially phenolic) and biological activities are variable by genetics (genus, species and cultivars) and environmental conditions i.e. plant age, field location and season (Forbes *et al.*, 2010; Izzreen and Fadzelly, 2013; Aralas *et al.*, 2014). In another study on plant with methanolic, ethanolic and aqueous extracts for phytochemical estimation of TFC, it is reported that aqueous extract has high level TFC which is agreed with our results, that in aqueous extracts of shortlisted active plants (*G. wallichianum* and *A. alpinum*) under study, have high TFC followed by EF in *G. wallichianum* and *A. alpinum* while high in EF of *E. parvifolia* followed by water fraction (Devika *et al.*, 2016). The fractionation showed the highest extract of TFC, and TFC in aqueous and ethyl acetate fractions impaired potent hepatoprotective effect, attributed the high antioxidant activity (Preethi *et al.*, 2016; Mhalla *et al.*, 2018).

To know the quality of phytoconstituents present in these three active hepatoprotective plants, HPLC and LCMS was performed. Development of HPLC methodology rendered a quick analysis for the detection of phenolic and flavonoid compounds in three plants (*G. wallichianum*, *E. parvifolia* and *A. alpinum*) extracts in methanol and their more active fractions (EF, BF and AF) respectively (table 4.12). Moreover, it also provided with good separation of the individual peaks of standards used (figure 4.54) and for tested samples (figures 4.55-4.65) respectively. Phenolic and flavonoids present in the crude methanolic extract of *G. wallichianum* roots (GWME) and its active fractions (GWEF, GWBF and GWAF) are shown in figures 4.55-4.58 respectively. HPLC chromatogram of crude methanolic extract (GWME) revealed that ten phenolic compounds are present (figure 4.55). There were fourteen phenolic compounds detected in GWEF and GWBF fraction (figures 4.56 and 4.57) respectively. Similarly, from figure 4.58 it is evident that chromatogram of GWAF revealed the presence of 13 phenolic compounds. Higher number of phenolic compounds were identified in GWEF and GWBF (14 each) followed by GWAF (13) and GWME (10). The presence of phenolic and flavonoids compounds in the crude methanolic extract of *E. Parvifolia* fruits (EPME) and its active fractions (EPEF and EPAF) are represented by peaks as shown in figures 4.59 - 4.61 respectively. HPLC chromatogram of crude methanolic extract (EPME) revealed the presence of nine phenolic

compounds (figure 4.59). Similarly, figure 4.60 showed chromatogram of EPEF fraction having twelve phenolic compounds and EPAF showed the presence of 10 phenolic compounds (figure 4.61). The order of presence of flavonoid compounds is; ethyl acetate fraction (12) > aqueous fraction (10) >crude methanolic extract (9) as evident from the HPLC chromatograms (figures 4.59- 4.61). Earlier, no any phytochemical constituent was identified from *E. parvifolia* but another species i.e.*E. Umbellata* (Thunb) have been studied, collected from Kalam; Malakand Division Khyber Pakhtunkhwa (KPK) Pakistan in which methanolic extract and sub fractions of fruits was analyzed by HPLC system. Earlier, by HPLC method, 12 compounds i.e. vitamin C, malic acid, gallic acid, epigallocatechin gallate, chlorogenic acid, morin, rutin, elagic acid, catechin hydrate, quercetin, pyrogallol and mandelic acid) are reported to identified from methanolic extract and eight compounds i.e. chlorogenic acid, epigallocatechin gallate, quercetin, morin, rutin, ellagic acid, pyrogallol and catechin hydrate) from chloroform extract and five compounds including rutin, quercetin, gallic acid, pyrogallol and mandelic acid from ethyl acetate extract from another medicinal plant (Nazir *et al.*, 2018). HPLC chromatogram of crude methanolic extract of *AA* (AAME) showed the presence of 7 phenolic compounds (figure 4.62). The figure 4.63 shows chromatogram of AAEF where ten phenolic compounds were detected. Similarly, fourteen phenolic compounds were detected in AABF (figure 4.64), while it is evident that chromatograms of AAAF showed 12 phenolic compounds (figure 4.65). The order of presence of phenolic and flavonoids in *AA* is n-butanol fraction (14) >aqueous fraction (12), ethyl acetate fraction (10) > methanolic extract (7). *A. alpinum* has antioxidant and anti-inflammatory properties. These compounds having high biological activities with low toxicity are; glycoside, astragalin, avicularin, quercetin-3,7-diglucoside, quercetrin, hyperoside, rutin, myricitin, kaempferol, quercetin and glycones that were known from aerial parts of *A. alpinum* (Vysochina Khramova, 2010). There is no data record about compounds identified from roots of *A. alpinum*. Thirteen phenolic acids (chlorogenic, gallic acid, vanillic acid,caffeic acid, gentsic acid, sinapic acid, syringic acid, anisic acid, ferulic acid, p-coumaric acid, rosmarinic acid, salicylic acid and cinnamic acid) in compound herbal medicines are reported to separated by HPLC in another study (Wen *et al.*, 2007).Some of the phytochemical constituents were identified by Saleem *et al.*, (2014) from aqueous methanolic extract of *Rumex dentatus* plant

(which also belongs to polygonacea family) by HPLC method which included chlorogenic acid, quercetin, myrecitin, vitamin C and kampferol that have known hepatoprotective effects (Song *et al.*, 2003; Janbaz *et al.*, 2004; Matic*et al.*, 2013; Rashed *et al.*, 2013; Saleem *et al.*, 2014). Compounds i.e. gallic acid, rutin, catechin, vanillic acid, 3,4 dimethoxy benzoic acid and quercetin had been identified by HPLC method from *Asparagus alba* leaves separated by hot aqueous extract that has strong antioxidant and free radical scavenging activity as well as hepatoprotective effect against CCl_4 induced liver damage due to presence of these phytoconstituents (Serairi-Beji *et al.*, 2018). Phytochemicals (phenolic and flavonoids) were previously identified from *chickpea* cultivars by Mekky *et al.*, (2016) by RP-HPLC-MS/MS in which hepatoprotective effect was attributed to ferulic acid, naringenin and kaempferol derivatives. LC-MS/MS method is reliable analytical technique to identify the unknown compounds from a mixture of plants extract. Compounds identified by LCMS-QTOF analysis of short listed most active plants (*G. wallichianum*, *E. parvifolia* and *A. alpinum*) are represented in the tables 4.13 – 4.15 respectively.

Total sixteen polyphenolic compounds (syringic acid, gallic acid, gallic acid dimer, protocatechuic, kaempferol, quercitrin, rutin, quercetin glucoside (hyperoside), apigenin, epicatechin, catechin, quinic acid, quercetin, chlorogenic acid, vanillic acid and caffeic acid) have been identified in crude methanolic extracts and most active fractions of these three plants extracts. The poly phenolic compounds identified by LCMS-QTOF analysis of *G. wallichianum*; higher number of compounds were identified in GWAF (11) followed by GWEF (9) and GWME (7) respectively (table 4.13). Similarly, the order of presence of compounds is EPEF (12) followed by EPME (7) and EPAF (4) respectively (table 4.14). Number of phenolic compounds identified were higher in AAAF = AAEF (15) and AAME (9) respectively (table 4.15). Results revealed that higher number of compounds are present if polar fractions of our studied plants, which is also supported by studies performed by Mekky *et al.*, (2016). The limit of detection (LOD) of phenolic compounds shows that most abundant compounds in the extracts found wererutin and quercetin (table 4.16, figures 4.66 and 4.67). Metabolic profile of samples was recorded at 280 nm (figure 4.68). Total twenty-three (23) polyphenolic and flavonoids compounds were identified from methanolic extracts and fractions, by HPLC and LCMS analysis from our three most active plants (*GW*, *EP* and

AA). These include 14 compounds identification by HPLC of which four phenolic acids (gallic acid, chlorogenic acid, ferulic acid and caffeic acid), nine flavonoids (epicatechin, luteolin, hyperoside, fisiten, apigenin-7-o-glucoside, rutin, naringenin, chrysin and apigenin) and one was benzene derived phenolic compound (benzene-triol), identified by co-elusion and retention time with reference standards (table 4.12). Seven (7) of them (gallic acid, caffeic acid, chlorogenic acid, epicatechin, hyperoside, ruitn and apigenin) present mostly in AF, EF and ME were further confirmed along with nine (9) more phenolic compounds (gallic acid dimer, quercitrin, kaempferol, protocatechuic acid, vanillic acid, syringic acid, quinic acid, catechin, quercetin) identification by LCMS in methanolic extracts and highly active fractions (EF and AF) in our studied most active plants for hepatoprotective effect. MS/MS spectra of twelve (12) identified compounds (gallic acid, protocatechuic acid, kaempferol, quercitrin, rutin, epicatechin, catechin, quinic acid, chlorogenic acid, quercetin, caffeic acid and vanillic acid) are indicated in figures 4.69 – 4.80. Phenolic compounds and elagic acid have been detected in variable quantity in different extracts of *G. robertianum* (Fodorea *et al.*, 2005; Neagu*et al.*, 2013; Kobakhidze and Alaniya, 2004). In another study, LCMS was performed to identify phytoconstituents in which four phenolic acids; β-resorcylic acid, caffecic acid, protocatechuic acid and 4-hydroxyphenylacetic acids and two flavonoids (trifolin and astragalin) were identified responsible for hepatoprotective effects from ethyl acetate and dichloromethane fraction of *Malvaviscus arboreus* ethanolic extract (Abdelhafez *et al.*, 2018). Regarding the phenolic and flavonoids components in wild blackberry; twenty-three different fruit samples were investigated by LCMS-QTOF analysis and was revealed the presence of thirty-four phenolic compounds which were correlated to antioxidant activity of analyzed samples and identification was based on retention time and data of mass spectra with standards in addition to data published (Oszmianski *et al.*, 2015). Phenolic compounds like gallic acid, hydroxyl benzoic acid, cumaric acid, vallinic acid and flavonoids like rutin, quercetin, kampferol and luteolin were identified by LCMS from *Zingiber zerumbet* (L) that have strong antioxidant activitiy (Kothandaraman and Shanmugam, 2018). Phenolic acid such as protocatechuic acid, caffeic acid and 4-hydroxyphenylacetic acid and two flavonoid glycosides; astragalin and trifolin extracted from ethyl acetate fraction have known hepatoprotective effects (Achyilla *et al.*, 2003; Carini *et al.*, 1992). It is formerly reported

that potential hepatoprotective effects of protocatechuic, rutin and caffeic acid are due to antioxidant and free radical scavenging potentials (Rejaie *et al.*, 2013; Zhong *et al.*, 2014; Abdelhafez *et al.*, 2018). Another study has revealed that plants having high contents of phenolics and flavonoids compounds exhibit potent hepatoprotective effects due to high antioxidant activity (Sabir *et al.*, 2008; Jain *et al.*, 2008, Jain *et al.*, 2011; Abdelhafez *et al.*,2018). Phytoconstituents present in medicinal plants impair the hepatoprotective effects against liver damage induced by chemicals and drugs (Sharma *et al.*, 2011; Rehman *et al.*, 2012). Rutin and quercetin have potential hepatoprotective effect against CCl_4 induced liver toxicity in mice having antioxidant and anti-inflammatory activities (Domitrovic *et al.*, 2012). Quercetin is known for biological, medicinal and pharmacological activities because of antioxidant activity (Afaf *et al.*, 2017). Quercetin has hepatoprotective effects against paracetamol-induced toxicity in mice (Afaf *et al.*, 2017; Barros *et al.*, 2017) and in our study; it may have heprotective effect along with other identified phytoconstituents. Strong antioxidant flavonoid has potential effect against melanoma cell (Hong *et al.*, 2013). Fan *et al.*, (2015) identified five phenolic compounds; ferulic acid, kampferol, quercetin, rutin and isorehamnetin from *Asparagus officinalis* that have strong antioxidant and antimicrobial activities. Rutin which is an important flavonoid having potential hepatoprotective effects on CCl_4 induced toxicity in mice (Ashraf *et al.*, 2012). Syringic acid and vallinic acid from *Lantina edodes* (edible mushroom) have played potential hepatoprotective effects against Concanavalin induced liver injury in mice (Itoh*et al.*, 2009). Caffeic acid has proven anti-inflammatory, ant carcinogenic, antibacterial and anti-mutagenic effects due to strong antioxidant activity (Mattos *et al.*, 2015). Our study for identification of compounds from three plants extracts is also supported by discussions of above findings. Hepatoprotective activity of quercetin, kaempferol and chlorogenic acid (Saleem *et al.*, 2014), caffeic acid (Yang*et al.*, 2013; Abdelhafez *et al.*, 2018), protocatechuic acid (Achyilla *et al.*, 2003; Abdelhafez *et al.*, 2018) and rutin (Hadriche *et al.* 2016; Abdelhafez *et al.*, 2018) are already known which also strongly support current study about hepatoprotective effects of three short listed plants extracts against CCl_4 liver damaged mice. High antioxidant activity as well as scavenging effects of these phytoconstituents against free radicals (DPPH) is strong evidence for detoxification of metabolites generated during CCl_4 induced toxicity in mice that

attributed to hepatoprotective effect of short listed active plants under this study. Hepatoprotective effect and antioxidant activity of phytoconstituents such as ferulic acid, epicatechin, hyperoside, fisetin, naringenin, apigenin, benzene-triol, gallic acid dimer and catechin identified in our study plants has not been known earlier on CCl_4 induced hepatotoxicity in mice. This hepatoprotective effect against CCl_4 induced liver damage in mice in our study was due to synergistic or combined effects of these phytoconstituents identified by HPLC and LCMS. During the whole experimental analysis, it is clearly proved that our studied plants rich in polyphenolic and flavonoids having strong antioxidant activity that were identified by HPLC and LCMS; attributed the most effective hepatoprotective role on CCl_4 induced liver damages in mice.

5.2. Summary

Serum biochemicals (ALT, AST, ALP and TB) are key biomarkers for diagnosis of liver disorders. When there is damage of organs like liver then these biochemical parameters are increased (Sallie *et al.*, 1991; Gupta *et al.*, 2006; Grace-lynn 2012; Afzal *et al.*, 2013; Sayed *et al.*, 2014; Hussain *et al.*, 2014; Serairi-Beji *et al.*, 2018). Hepatoprotective agents are expected to reduce the elevated levels of serum parameters i.e ALT, AST, ALP, and TB. Plants extract accelerate the liver regeneration and reduce serum enzymes leakage into circulation. From all the parameters investigated and discussed above for the results of our study, it can be correlated that the selected plants extracts have potential hepatoprotective effect against CCl_4 toxicity induction in mice. Acute oral toxicity test of selected plants extract showed that these plants have no toxic effects and they are safe for biological activity. The lethal dose (LD_{50}) for mice of these plants extract was beyond the 2000 mg/kg. Low ($1/10^{th}$) and high dose ($1/5^{th}$) level of tolerated dose (2000mg/kg) was further applied to study the hepatoprotective effects of crude methanolic extract of selected plants. The CCl_4 induction caused the liver injury which is proved by the elevation in the level of liver marker i.e. ALT, AST, ALP, and TB in the serum (figures 4.1 – 4.4) and histopathological changes in liver like necrosis of hepatocytes, fatty changes and dilated sinusoid, inflammatory cells infiltration, bleeding area in the hepatic lobes with the loss of cellular architecture due to CCl_4 intoxication in toxicant control group (figure 4.6) as compared to normal mice (G-I) in

figure 4.5. When mice were treated with SD (100mg/kg) and crude methanolic extract of selected plant with 200mg/kg and 400mg/kg, then the elevated level of liver maker enzymes was significantly reduced (figures 4.1 – 4.4 and table 4.2) as compared to toxicant control group attributed to improvement of liver cellular structures with regeneration, less necrosis and normal central vein as shown in figures 4.8 (a, b) – 4.17 (a, b). The protective effect of selected plants extract was dose dependent with variability of some plants as compared themselves. The order of protective effect of these plants was AAME > GWME > EPME >VCME > ODME > RBME > PMME > FNME > TSME > DRME. The most active hepatoprotective short listed plants (*G. wallichianum* roots, *E. parvifolia* fruits and *A. alpinum* roots) were fractionated on increasing polarity basis of solvents for further investigation about antioxidant activity and phytochemical composition as well as hepatoprotective effects to establish an idea about the most active hepatoprotective fractions and nature of phytochemicals that attributed hepatoprotective effect on CCl_4 intoxication. The results of antioxidant activity showed that DPPH scavenging effect of extract increased when the dose was increased and the antioxidant activity was highest of those fractions having low IC_{50} value of these three plants extract/fraction as compared to standard ascorbic acid (IC_{50}). The highest antioxidant activity of *G. wallichianum* extract/fraction was of aqueous fraction followed by ethyl acetate and n-butanol fraction. The highest antioxidant activity of *E. parvifolia* extract/fraction was of EPAF followed by EPEF and EPME and the highest antioxidant activity of *A. alpinum* extract/fraction was of AAAF followed by AAEF, AABF and AAME. The antioxidant activity was due to presence of different phytochemicals such as phenolic and flavonoids contents in extract/fraction of these three plants under study. The most active fractions for reducing the elevated levels of liver biochemical markers i.e. ALT, AST, ALP and TB in serum due to CCl_4 intoxication were AF, EF and BF of *G. wallichianum* and *A. alpinum* and AF, EF of *E. parvofolia* which were supported by the histopathological analysis of liver of mice in different treated groups which showed more regeneration, normal cellular structure and intact central vein as well as no necrosis which were comparable to normal control mice and standard drug (SD) treatment. The effect was due to nature of distribution of phytochemicals in solvents and dose dependent as well. The most active fractions of these three plants were observed for high antioxidant activity and

composition of polyphenolic and flavonoids that were identified by HPLC and LCMS. The phytoconstituents have high antioxidant activity due to which liver was protected against CCl_4 intoxicated resulted, ROS and oxidative stress. These compounds were never identified earlier from crude methanolic extracts and different fractions in these three most active plant for hepatoprotective effect. The *G. wallichianum* root extract has highest significant hepatoprotective effect in aqueous fraction followed by ethyl acetate fraction and n-butanol fraction as compared to other fractions of this plant similarly there was highest significant hepatoprotective effect of *E. parvifolia* fruits extract in aqueous fraction followed by ethyl acetate fraction. The highest significant hepatoprotective effect of *A. alpinum* root extract was demonstrated in aqueous fraction followed by ethyl acetate fraction and n-butanol fraction. The hepatoprotective effect of most active fractions was attributed to the presence of compounds identified by HPLC and LCMS analysis. Consequently, hepatoprotective potential of *G. wallichianum*, *E. parvifolia* and *A. alpinum* could be related to synergistic interaction of phytochemicals and combined effects. The hepatoprotective effect of these plants is correlated to strong antioxidant activity with high scavenging effect to free radicals generated due to CCl_4 intoxication. This correlation of antioxidant activities and hepaptoprotective effects is attributed to polyphenolic and flavonoids contents identified by HPLC analysis (table 4.12) of different extracts/fraction that include four phenolic acids (gallic acid,ferulic acid, chlorogenic acid and caffeic acid), nine flavonoids (epicatechin, luteolin, hyperoside, rutin, fisten, naringenin, apigenin-7-o-glucoside, chrysin and apigenin) and one was benzene derived phenolic compound (benzene-triol) of which seven (7) of them (gallic acid, caffeic acid, chlorogenic acid, epicatechin, hyproside, ruitn and apigenin) were further confirmed by LCMS analysis mostly from AF, EF and ME in addition to gallic acid dimer, quercitrin, kaempferol, protocatechuic acid,vanillic acid (in *AA*), syringic acid, quinic acid (in *GW*), catechin, quercetin (in *EP*except kaempferol, syringic acid and vanillic acid) phytoconstituents identified by LCMS (table 4.13-4.15).

5.3. Conclusions

The plants under our study are non-toxic than other synthetic compounds practically. Major avenue for discovery of new drugs is achieved by screening of natural products from

plant sources. The main endeavor was to investigate hepatoprotective effect of selected plants extracts by evaluating the effects on various liver biochemical parameters. Based on outcomes it is concluded that: the selected plants showed hepatoprotective effects against CCl_4 induced toxicity in experimental animals. Their effect is dose and time dependent which is proved by biochemical analysis of liver biochemicals and liver histopathology as compare to toxicant control mice. The most active hepatoprotective plants (*G. wallichianum*, *E. parvifolia* and *A. alpinum*) have highest effect shown by their intermediate polar and more polar solvent fractions (*EF*, *BF* and *AF*) in experimental animals. The selected plants extracts have markedly decreased the elevated concentrations of liver biochemical/markers (ALT, AST, ALP, and TB) intoxicated with CCl_4 in mice which strongly recommends their hepatoprotective activity and this hepatoprotective effect may be due to mechanism of inhibition of reactive oxygen species and improvement of antioxidant level in dose dependent manner. The extracts have improved the hepatic regeneration, protected the hepatocytes and stopped necrosis formation, as observed during histopathological analysis. There was not any study conducted before in terms of their antioxidant and hepatoprotective effect. The root cause of hepatoprotective effect is antioxidant effect of plants crude methanolic extract and their fractions as well. The active plants shortlisted and studied for antioxidant activity and hepatoprotective effects after solvent-solvent partition into fractions (HF, CF, EF, BF and AF) showed that these plants are rich in polyphenolic and flavonoids phytoconstituents (identified by HPLC and LCMS) in the different extracts/fractions that played the synergistic effect to protect liver against CCl_4 induced toxicity due to antioxidant activity and scavenging effect to free radicals generated during CCl_4 intoxication. There was not any phytoconstituent identified earlier to this study from these short listed most active plants (*G. wallichianum*, *E. parvifolia* and *A. alpinum*) of Poonch division of AJ and K, Pakistan with crude methanolic extract and other solvent fractions. These compounds may improve the management of liver disorders. The scientific evidence obtained in this way is helpful for preparations of herbal medicines to develop drugs in sense of modern medicines for management of liver treatment.

5.4. Recommendations

It is an important aspect to use the medicinal plants for prevention/treatment of diseases like hepatotoxicity. The most population of the world is dependent on medicinal plants to treat various ailments. Use of plants is more beneficial because they are less toxic than synthetic compounds of modern drugs. The screening of plants represents an important avenue for drug discovery. Liver disorders management by natural herbal drugs is still a great challenge for health professionals. Selected plants in our study from Poonch division of Azad Jammu and Kashmir, Pakistan were still not reported for antioxidant activity and pharmacological investigations. This study would helpful for the safe use of these plants to treat different disease by local community.

- The phytochemicals like polyphenolic and flavonoids are richly present in roots of *G. wallichianum*, *A. alpinum* and fruits of *E. parvifolia* are responsible for antioxidant activity attributed to hepatoprotective effects.
- These extracts can be used with other antioxidants.
- The phytoconstituents may be isolated and characterize to appraise their chemistry and identify which particular ingredient has hepatoprotective effect from each plant.
- The roots of *G. wallichianum*, *A. alpinum* and fruits of *E. parvifolia* can be used for liver treatment.
- Intermediate polar solvent (ethyl acetate) and more polar solvents (n-butanol, methanol and water) separate the phytochemicals efficiently from *G. wallichianum* roots, *A. alpinum* roots and *E. parvifolia* fruits as compare to less polar fractions and may be used for extraction of phytoconstituent of plants.
- More pharmacological evaluations can be performed against other diseases like cancer and diabetes.
- Exact cellular mechanism of action and molecular aspects of phytochemical can be assessed.

- Other plant parts of *G. wallichianum*, *E. parvifolia*and *A. alpinum* can be assessed for hepatoprotective effects.

Selected plants other than short listed most active plant (*G. wallichianum*, *E. parvifolia* and *A. alpinum*) may be further assessed for other pharmacological activities to know their antioxidant potential and mechanism of action.

REFERENCES

6. REFERENCES

Abbasi AE., Arshad M., and Saboor A. Ethno-botanical appraisal and medicinal uses of plants in Patriata New Murree - Evidence from Pakistan. Journal of Ethno-botany and Ethno-medicine. 2013, 9 (1):1-3.

Abdelhafez OH., Fawzy MA., Fahim JR., Desoukey SY., Krischke M., and Mueller MJ. Hepatoprotective potential of *Malvaviscusarboreus* against carbon tetrachloride-induced liver injury in rats. PLoS ONE. 2018, 13(8):1-18. e 0202362. https: // doi.org /10.1371 /journal. P one.0202362.

Abdullah., Khan MA., Ahmad W., Ahmad M., and Nisar M. Hepatoprotective effect of the solvent extracts of *Viola canescens* Wall. ex. Roxb. against CCl_4 induced toxicity through antioxidant and membrane stabilizing activity. BMC Complementary and Alternative Medicine. 2017, 17 (10): 1-11. DOI 10.1186/s12906-016-1537-7.

Aboelsoud NH. Herbal medicine in ancient Egypt.Journal of Medicinal Plants Research. 2010, 4(2): 082-086.

Abou-Ela M., El-Shaer N., and Abd El-Azim T. Chemical constituents and hepatotoxic effect of the berries of Juniperus phoenicea Part 2. Nat Prod Sci. 2005, 11(4): 240-247.

Achyilla GS., Kotgale SG., and Wadodka AK. Hepatoprotective activity of *Panchgavya gritha* in CCl_4- induced hepatotoxicity in rats. Indian J Pharmacol. 2003, 35: 311-315.

Adeel M., Malik N.R., Shinwar KZ, and Mahmood A. Ethnobotanical survey of plants from Neelum Azad Kashmir. Pakistan Journal of Botany. 2011, (43):105-110.

Adeneye AA., Olagunju JA., Banjo AAF., Abdul SF., Sanusi OA., Sanni OO., Osarodion BA., and Shonoiki OE. The aqueous seed extract of Carica papaya Linn. Prevents carbon tetrachloride induced hepatotoxicity in rats. International Journal of Applied Research in Natural Products. 2009, 2 (2):19-32.

Afaf A., Faras EI, Amel L., and Elsawaf. Hepatoprotective activity of quercetin against paracetamolinduced liver toxicity in rats. Tanta Medical Journal.2017, 45: 2.

Afzal U., Gulfraz M., Hussain S., Malik F., Maqsood S., Shah I., and Mahmood S. Hepatoprotective effects of *Justicia adhatoda* L. against carbon tetrachloride (CCl_4) induced liver injury in Swiss albino mice. African Journal of Pharmacy and Pharmacology. 2013, 7(1): 8-14.

Agarwal SS., Development of hepatoprotective formulations from plant sources. Pharmacology and Therapeutics in the New Millennium. Edited by Gupta SK, Narosa Publishing House, New Delhi. 2001, 357-358.

Agbafor KN., Ogbanshi ME., and Akubugwo EI. Phytochemical screening, hepatoprotective and antioxidant effects of leaf extracts of *Zapoteca portoricensis.* Advances in Biological Chemistry. 2014, 4: 35-39.

Agbor AG., and Ngogang YJ. Toxicity of herbal preparations. Chemical Journal of Ethno-botany.2005, 1: 23-28.

Aghel N., Rashidi I., and Mombeini A. Hepatoprotective Activity of *Capparis spinosa* Root Bark against CCl_4 Induced Hepatic Damage in Mice. Iran. J. Pharm. Res. 2007, 6(4):285-290.

Ahmad A., Pillai KK., Najmi AK., Ahmad SJ., Pal SN., and Balani DK. Evaluation of hepatoprotective potential of jigrine post-treatment against thioacetamide induced hepatic damage. J. Ethnopharmacol. 2002, 79 (1): 35-41.

Ahmad B., Ismail M., Iqbal Z., and ChoudharyIM. Biological activities of *Geranium wallichianum*. Asian Journal of Plant Sciences.2003, 2 (13): 971-973.

Ahmad M., Shah A.S., Khan R.A., Khan FU., Khan NA., Shah MS., and Khan MR. Antioxidant and antibacterialactivity of crude methanolic extract of Euphorbia prostrate collected from District Bannu (Pakistan). African Journal of Pharmacy and Pharmacology. 2011, 5: 1175-1178.

Ahmad SK., and Habib S. Indigenous knowledge of some medicinal plants of Himalayas region, Dawarian Village Neelum Valley, Azad Kashmir (Pakistan). Universal Journal of Plant Sciences. 2014, 2 (2): 40-47.

Ahmad SK., Kiyani KW., Hameed M., Ahmad F., and Nawaz T. Floristic diversity and ethno-botany of Sehnsa, District Kotli Azad Kashmir (Pakistan). Pakistan Journal of Botany. 2012a, 44: 195-201.

Ahsan R., Islam KM., Balbul IJ., Musaddik MA., and Haque E. Hepatoprotective Activity of Methanol Extract of Some Medicinal Plants Against Carbon Tetrachloride-Induced Hepatotoxicity in Rats. European Journal of Scientific Research. 2009, 37(2): 302-310.

Ajaib M., Shinwari K Z., Khan N., and Wahab M. Ethnobotanical studies on useful ahrubs of District Kotli, Azad Jammu & Kashmir (Pakistan). Pakistan Journal of Botany. 2010, 42 (3): 1407-1415.

Ajaib M., Khan Z., and Siddiqui FM. Ethnobotanical study of useful climbers/ twiners of District Kotli, Azad Jammu & Kashmir.International Journal of Biology &Biotechnology.2012,9 (4): 421-427.

Akhtar MS., Amin M., Ahmad M., and Alamgeer. Hepatoprotective effect of *Rheum emodi* Roots (*Revandchini*) and Akseer-e-Jigar against paracetamol-induced hepatotoxicity in rats. Ethno-botanical Leaflets. 2009,13: 310-315.

Albano E., Rundgren M., Harvison PJ., Nelson SD., and Moldeus P. Mechanisms of N-acetyl-p benzoquinone imine cytotoxicity. Mol Pharmacol. 1985, 28(3):306 -11.

Alisi CS., Onyeze GO., Ojiako OA., Osuagwu CG. Evaluation of the protective potential of Chromolaena odorata Linn. extract on carbon tetrachloride-induced oxidative liver damage. Int J Biochem Res Rev. 2011,1(3):69 - 81.

Almey AA., Khan CAJ., Zahir S., Suleiman KM., Aisyah MR., and Rahim KK. Total phenolic content and primary antioxidant activity of methanolic and ethanolic extracts of aromatic plants' leaves. Int Food Res J. 2010, 17:1077–84.

Alonso MG., Pascual-Teresa S., Santos-Buelga C., and Rivas- Gonzalo JC. Evaluation of antioxidant properties of fruits. Food Chem.2004, 84: 13-18.

Alshawsh MA., Abdulla MA., Ismail S., and Amin AZ. "Hepatoprotective Effects of *Orthosiphon stamineus* Extract on Thioacetamide-Induced Liver Cirrhosis in Rats,"

Evidence-Based Complementary and *Alternative Medicine*. 2011, 6 pages. Article ID 103039, doi:10.1155/2011/103039.

Amarowicz R. Tannins: The new natural antioxidants. Eur. J. Lipid Sci. Technol. 2007, 109: 549–551.

Amjad SM., Arshad M., and Qureshi AR. Ethno-botanical inventory and folk uses of indigenous plants from Nasoora National Park Azad Kashmir (Pakistan). Asian Pacific Journal Tropical Biomedicine. 2015, 5(3): 234-241.

Anees S., Dar KB., Bhat AH., Ahmad S., and Rabia Hamid R. Anti-hyperlipidemic and antioxidant capacity of active extracts of *Fragaria nubicola* in high fat diet fed hyperlipidemic rats. International Journal of Pharmaceutical Science and Research (IJPSR). 2018, 9(6): 2228-2237.

Aqil F., Ahmed I., and Mehmood Z. Antioxidant and free radicalscavenging properties of twelve traditionally used Indian medicinal plants. Turk J Biol. 2006, 30: 177-183.

Aralas S., Mohamed M., and Bakar MFA. "Antioxidant properties of selected salak (*Salacca zalacca*) varieties in Sabah, Malaysia," Nutrition and Food Science. 2014, 39 (3): 243–250.

Arfan M., and Kader A. Antioxidant properties-tannins from *Litsea monopetala*. Polish J Food and Nutr Sci.2006,34: 66-69.

Asadi-Samani M., Rafieian-Kopaei M., and Azimi N. Gundelia: A systematic review of medicinal and molecular perspective. Pak J Biol Sci. 2013, 16:1238-1247.

Asadi-Samani M., Farkhad KN., Azimi N., Fasihi A., Ahandani AE., and Kopaei RM. Medicinal plants with hepatoprotective activity in Iranian folk medicine. Asian Pacific Journal of Tropical Biomedicine.2015, 5(2):146-157.

Ashoush SI., El-Batawy IO., Gehan A., and El-Shourbagy. Antioxidant activity and hepatoprotective effect of pomegranate peel and whey powder in rats. Annals of Agricultural Science.2013, 58(1): 27–32.

Ashraf J., Nagma., Siddique J., MiraniN., and Rub A. Protective effect of rutin against carbon tetrachloride-induced hepatotoxicity in mice. International Journal of Drug Development & Research. 2012, 4(2): 352-357.

Aslam M., Afzal N., Anis I., Khalid M., Hussain A., Bukhari T.H., Ibrahim M., Ali B., Niaz A.M., Chaudhry H., and Arshad M. Study on antioxidant activity of essential oil of *Thymus serphyllum*. Pakistan Journal of Chemicals.2012, 2(1):1- 4.

Asselah T., Bieche I., Sabbagh A., Bedossa P., Moreau R., Valla D., and Marcellin P. Gene expression and hepatitis C virus infection. Gut.2009, 58(6): 846-858.

Atiq M., Gill M.L., and Khokhar N. Quality of life assessment in Pakistani patients with Chronic liver disease. Journal of Pakistan Medical Association.2004, 54(3):113-115.

Attia HA., Soad M., Nasr., Jalil HA., Almaweri1., Doaa Sedky., Amany M., Mohamed., Hassan M., Desouky., Mostafa A., and Shalaby. Phytochemical, antioxidant and hepatoprotective effects of different fractions of Moringa oleifera leaves methanol extract against liver injury in animal model. Asian Pacific Journal of Tropical Medicine. 2018, 11(7):423-429.

Aziz S., and Rehman H. Studies on chemical constituents of *Thymus serphyllum*. Turk Journal of Chemistry. 2008, 32: 605-614.

Bansal AK., Bansal M., Soni G., and Bhatnagar D. Protective role of Vitamin E pre-treatment on N-nitrosodiethylamine induced oxidative stress in rat liver.Chem Biol Interact. 2005,156(2-3):101-11.

Barkatullah., Ibrar M., and Muhammad N. Evaluation of Zanthoxylum armatum DC for in-vitro and in-vivo pharmacological screening. African Journal of Pharmacy and Pharmacology. 2011, 5(14):1718-1723.

Barros PP., Silva GH., Gonçalves GMS., Oliveira JC., Pagnan LG., FlexaLA.Hepatoprotective Effect of Quercetin Pretreatment Against Paracetamol-Induced Liver Damage and Partial Hepatectomy in Rats. Braz Arch Biol Technol. 2017, 60: Pages 10. e17160138.

Bellini MFJPF., Angeli R., Matuo AP., TerezanLR., Ribeiro., and Mantovani MS. Antigenotoxicity of *Agaricus blazei* mushroom organic and aqueous extracts in chromosomal aberration and cytokinesis block micronucleus assays in CHOk and HTCcells. Toxicology in Vitro. 2006, 20 (3): 355–360.

Bergendi L., Benes L., Durackova Z., and Ferencik M. Chemistry, physiology and pathology of free radicals. Life Sciences. 1999, 65(18-19):1865-1874.

Bertoncelj J., Doberšek U., Jamnik M., and Golob T. Evaluation of the phenolic content, antioxidant activity and colour of Slovenian honey. Food Chem.2007, 105: 822-828.

Bhattarai S., Chaudhary RP., and Taylor RSL. Screening of selected Ethno-medicinal plants of Manang District Central Nepal for antibacterial activity. Ethno-botany.2008, 20: 9-15.

Bhawna S., and Kumar US. Hepatoprotective activity of some indigenous plants. International Journal of Pharmacy and Technological Research.2010, 2 (1): 568-572.

Bilzer M., Roggel F., and Gerbes A. Role of Kupffer cells in host defense and liver diseases, Liver International.2006, 26(10):1175-86.

Bjelakovic G., Nikolova D., Gluud LL., Simonetti RG., and Gluud C. Mortality in randomized trials of antioxidant supplements for primary and secondary prevention; systematic review and meta-analysis. J Amr Med Med Ass (JAMA).2007, 297(8): 842-857.

Bokhari TZ., Raja R., Younis u., Bushra E. and Ummara U. Medicinal importance of few plants from Azad Jammu &Kashmir, Pakistan.Fuuast j. Biol. 2013, 3(1): 93-99.

Boligon AA., and Athayde ML. Importance of HPLC in Analysis of Plants Extracts. Austin Chromatogr. 2014; 1(3): 2.

Bolling BW., Dolnikowski G., Blumberg JB., and Chen CY. Polyphenol content and antioxidant activity of California almonds depend on cultivar and harvest year. Food Chem. 2010, 122: 819-25.

Bouasla I., Bouasla A., Boumendjel A., Messarah M., Abdennour C., Boulakoud MS., *et al.* *Nigella sativa* oil reduces aluminium chloride-induced oxidative injury in liver and erythrocytes of rats. Biol Trace Elem Res. 2014, 162(1–3): 252-261.

Bozkurt E., Atmaca H., Kisim A., Uzunoglu S., Uslu R., and Karaca B. Effect of *Thymus serphyllum* extract on cell proliferation, apoptosis and Epigenetic events in human breast cancer cells. Nutrition and Cancer.2012, 64(8):1245-1250.

Briskin DP. Medicinal plants and phytomedicines, linking plant biochemistry and physiology to human health. *Plant Physiol.*2000,124: 507-514.

Bruneton J. "Pharmacognosie, phytochimie, Plantes medicinales'' (2nd edition). Tec et Doc., Lavoisier, Paris.1993, 915.

Butt AS. Epidemiology of Viral Hepatitis and Liver Diseases in Pakistan. Euroasian J Hepato-Gastroenterol. 2015, 5(1): 43-48.

Butt K., Yunas S., andSheikh RM. Hepatoprotective effect of *Cichoriumintybus* on paracetamol induced liver damage in albino rats. Libyan Agriculture Research Center Journal International.2012, 3(2): 60-63.

Buyukokuroglua M., GülçIn I., Oktayc M., and KüfrevIoglu Ö. *In vitro* antioxidant properties of dantrolene sodium. Pharmacological Research.2001, 44(6), 491-494.

Cai Y., Luo Q., Sun M., and Corke H. Antioxidant activity and phenolic compounds of 112 traditional Chinese medicinal plants associated with anticancer. Life Sci. 2004, 74(17):2157-84.

Carini R., Comoglio A., Albano E., and Poli G. Lipid peroxidation and irreversible damage in the rat hepatocytes model: protection by the silybin-phospholipid complex. Biochem Pharmacol, 1992, 38: 2859-2865.

Céspedes CL., Hafidi EM., Pavon N., and Alarcon J. Antioxidant and cardioprotective activities of phenolic extracts from fruits of Chilean blackberry *Aristotelia chilensis* (Elaeocarpaceae), Maqui. Food Chem. 2008,107: 820- 829.

Ch MI., Ahmad F., Maqbool M., and Husain T. Ethno botanical inventory of flora of Maradori Valley District Forward Kahuta Azad Kashmir, Pakistan. American Journal of Research Communication. 2013, 1(6): 239-261.

Chand N., Durrani FR., Ahmad S., and Khan A. Immunomodulatory and hepatoprotective role of feed-added Berberis lycium in broiler chicks. J Sci Food Agric.2011,91(10): 1737-1745.

Chand T., Bhandari A., Bhupendra K., Kumawat., Basniwal P., Sharma S., and Verma R. In vitro antioxidant activity of alcoholic extract of seed of *Cucumis callosus* (Rottl.) cogn. American Journal of Pharmtech Research. 2012, 2 (3): 2249-3387.

Chandan BK., Saxena AK., Shukla S., Sharma N., Gupta DK., Singh K *et al.* Hepatoprotective activity of *Woodfordia fruticosa* Kurz flowers against carbon tetrachloride induced hepatotoxicity. J Ethnopharmacol. 2008,119(2): 218-224.

Chandan BK., Saxena AK., Shukla S., Sharma N., Gupta DK., Suri KA *et al.* Hepatoprotective potential of *Aloe barbadensis* Mill. Against carbon tetrachloride induced hepatotoxicity. J Ethnopharmacol. 2007, 111: 560-6.

Chander TR., Reddy YN., Bollikunta V., and Warangal. Evaluation of Hepatoprotective and Antihepatotoxic Activity of Ethanolic Extract of *Evolvulus alsinoides* Linn on CCl_4 Induced Rats. Asian Journal of Pharmacology and Toxicology. 2014, 02 (04): 01-06.

Chen Y., Wang G., Wang H., Cheng C., Zang G. Phytochemical Profiles and Antioxidant Activities in Six Species of Ramie Leaves. PLoS ONE. 2014, 9(9):108140. doi:10.1371/ journal. P One. 0108140.

Cheng N., Ren N., Properties of vanillin in carbon tetrachloridetreated rats. Eur J Pharm. 2011, 668: 133-139.

Cortan., Ramzi S., Kumar., Vinay., Fausto., Nelson., Robinson., Stanley L., Abbas., and Robbinson AK. Pathologic Basis of Disease(7thedition.) St. Louis Elsevier Saunders. 2005, 45: 878.

Couladis M., Tzakou O., Verykokidou E., and Harvala C. Screening of same Greek aromatic plants for antioxidant activity. Phytother Res.2003,17:194-195.

Cragg MG., Newman DJ., and Snader KM. "Natural products in drug discovery and development." Journal of NaturalProducts. 1997, 60: 52–60.

Cravotto G., Baffo L., Genzini L., and Garella D. Phytotherapeutics: An evaluation of potential of 1000 plants, Journal of Clinical Pharmacology and Therapeutics. 2010, 35(1):11-48.

Cui X., Gu X., KangW. Antioxidant activity *in vitro* and hepatoprotective effects *in vivo* of compound *lobelia*. Afr J Tradit Complement Altern Med. 2016, 13(5):114-122.

Dia J., and Mumper RJ. Plant Phenolics: Extraction, analysis and their antioxidant and anticancer properties. Molecules.2010, 15: 7313–7352.

Dar EM., Cochard R., Shresth PR., and Sajjad A. Floristic composition of Machiara National Park District Muzaffarabad Azad Kashmir (Pakistan), International Journal of Biosciences (IJB). 2012, 2(4): 228-245.

Darwisd Y., Laaksoa I., and Hiltunen R. Chemical composition and in vitro antioxidative activity of a lemon balm (Melissa officinalis L.) extract. LWTFood Sci. Technol. 2008, 41: 391-400.

Devika M., Joshi H., and Nalini MS. Phytochemicals, Antioxidative and *in vivo* Hepatoprotective Potentials of *Litsea floribunda* (BL.) Gamble (Lauraceae) - An Endemic Tree Species of the Southern Western Ghats, India. Jordan Journal of Biological Sciences. 2016, 9: 163 – 171.

Dixit N., Baboota S., Kohli K., Ahmad S., Ali J. Silymarin: A review of pharmacological aspects and bioavailability enhancement approaches. Indian Journal of Pharmacology. 2007, 39 (4): 172-179.

Do QD., Angkawijaya AE., Tran-Nguyen PL., Huynh LH., Soetaredjo FE., Ismadji S., and Yi- Ju YH. Effect of extraction solvent on total phenol content, total flavonoid content,

and antioxidant activity of Limnophila aromatic. Journal of food and drug analysis. 2014, 22: 2 9 6-302.

Domitrovic R., Jakovac H., MarchesiV V., zević S VK., Cvijanović O., Tadić Z., and Rahelić D. Differential hepatoprotective mechanisms of rutin and quercetin in CCl_4-intoxicated BALB/cN mice. Acta Pharmacologica Sinica.2012, 33: 1260–1270.

Don C., and Rockey. Antifibrotic Therapy in Chronic Liver Disease. Clinical Gastroenterology and Hepatology. 2005, 3:95–107.

Dorji K., Tobgay S., and Yangdon N. The Ethno-botanical Studies of Medicinal and Aromatic Plants in Sakteng Wildlife Sanctuary, Trashigang, Bhutan. Int. J. Curr. Res. Biosci. Plant Biol.2017,4(4):75-82.

DuraipandiyanV., Ayyanar M., and Ignacimuthu S. Antimicrobial activity of some ethnomedicinal plants used by Paliyar tribe from Tamil Nadu, India. BMC Complementary Altern. 2006,6: 35-41.

Elberry AA., Harraz FM.., Ghareib SA., Gabr SA., Nagy AA., and Sattar EA. Methanolic extract of Marrubium vulgare ameliorates hyperglycemia and dyslipidemia in streptozotocin-induced diabetic rats. Int J Diabetes Mellit.2011, 11: 1877-1878.

Elhardallou SB. Cytotoxicity and biological activity of selected sudanese medicinal plants. Research Journal of Medicinal Plants.2011, 5: 201–229.

Escarpa A., and Gonzalez MC. Approach to the content of total extractable phenolic compounds from different food samples by comparison of chromatographic and spectrophotometric methods. Anal Chim Acta. 2001, 427:119–27.

Exarchou V., Nenadis N., Tsimidou M., Gerothanassis I.P., Troganis A., and Boskou D. Antioxidant activities and phenolic composition of extracts from Greek oregano, Greek sage and summer savory. J. Agric. Food Chem. 2002, 50: 5294-5299.

Fabricant SD., and Norman RS. The values of plants used in traditional medicine for drug discovery. Environment Health Prospect.2011,109: 69-75.

Fan R., Yuan F., Wang N., GaoY., and Huang Y. Extraction and analysis of antioxidant compounds from the residues of *Asparagus officinalis* L. J Food Sci Technol. 2015, 52 (5): 2690–2700.

Fan XH., Cheng YY., Ye ZL., Lin RC., and Qian ZZ. Multiple chromatographic fingerprinting and its application to the quality control of herbal medicines. Anal Chim Acta. 2006, 555(2): 217-224.

Feijoo M., Túnez I., Ruiz A., Tasset I., Muñoz E., and Collantes E. Oxidative stress biomarkers as indicator of chronic inflammatory joint diseases stage. Reumatología Clínica (English Edition). 2010, 6(2): 91-94.

Fodorea CS., Vlase L., SuciuS., Tamas M., and Leucuta S.E. Preliminary HPLC study on some polyphenols of *Geranium robertianum* L. (Geraniaceae). Rev. Med. Chir. Soc. Med. Nat. Iasi.2005, 109: 174–178.

Forbes CSB., Zhang Y., and Nair MG. "Anthocyanin content, antioxidant, anti-inflammatory and anticancer properties of blackberry and raspberry fruits." Journal of Food Composition and Analysis.2010, 23 (6): 554–560.

Fouwad., Mostafa A., Gamal A.M., Sabrin M.R.I., and Ehab S.E. Antioxidant and anti-inflammatory activities of phenolic constituents from *Primula elatior* aerial parts. International Journal of Pharmacognocy and Phytochemical Research.2014, 6(1):74 - 78.

Franco E., Meleleo C., Serino L., Sorbora D., and Zarati L. Hepatitis A; epidemology and prevention in developing countries. World Journal of Hepatology.2012, 4(3): 68-73.

Friedman LS. Liver, Biliary Tract and Pancreas Disorders, Current Medical Diagnosis and Treatment. NewYork, Mc Graw Hill, Chapter16, http:// accessmedicine. mhmedical. com/ content. aspx bookid=330&Sectionid.2014, 44291018.

Gaikwad AS., Gayatri S., Kamble., Devare S., Nirmala R., Deshpande., Jyoti P., and Salvekar. In vitro evaluation of free radical scavenging potential of Cassia auriculata L. J Chem Pharm Res. 2011, 3(4): 766-772.

Galicia-Moreno M., and Gutiérrez-Reyes G. "The role of oxidative stress in the development of alcoholic liver disease," Revista de Gastroenterologia de Mexico. 2014, 79(2): 135-144.

Ganapathi N., and Jamaludin M. Medicinal phytochemical and pharmacological properties of Kesum (*Polygonium minus* Linn.) A Mini Review. International Journal of Pharmacy and Pharmaceutical Sciences.2014, 6(4): 682-688.

Gbadegesin MA., Odunola OA., Akinwumi KA., and Osifeso OO. Comparative hepatotoxicity and clastogenicity of sodium arsenite and three petroleum products in experimental Swiss Albino Mice: the modulatory effects of *Aloe vera* gel. Food Chem Toxicol. 2009, 47(10):2454-7.

Gebhardt R. Oxidative stress, plant-derived antioxidants and liver fibrosis. Planta Medica.2002, 68(4): 289-296.

Genene B., and Hazare ST. Isolation and Characterization of Bioactive Compounds from Medicinal Plants of Ethiopia- A Review. Curr Trends Biomedical Eng & Biosci. 2017,7(5): 86-89.

Ghiasuddin., Rehman T., Arfan M., Liaqat L., Waliullah., Abdur rauf., Khan I., Mohammad G., and Choudhary I.M. In-vitropharmacological investigations of aerial partsof *Indigofera heterantha*. Journal of Medicinal Plants Research.2011, 5(24): 5750-5753.

Ghosh A., Ghosh T., and Jain S. Silymarin-A review on the pharmacodynamics and bioavailability enhancement approaches. Journal of Pharmaceutical Science and Technology. 2010, 348-355.

Gomes PCS., Seabra RM., Andrade PB., and Ferreira MF. Determination of phenolic antioxidant compounds produced by calli and cell suspensions of sage (*Salvia officinalis* L.). *J Plant Physiol*.2003,160:1025-1032.

Gong F., Yin ZH., Xu QT., and Kang WY. Hepatoprotective effect of *Mitragyna rotundifolia* Kuntze on CCl_4-induced acute liver injury in mice. Afr. J. Pharm. Pharmacol. 2012, 6: 330-335.

Grace-Lynn C., Chen Y., Latha LY., Kanwar JR., Jothy SL., Vijayarathna S., and Sasidharan S. Evaluation of the hepatoprotective Effects of Lantadene A, a pentacyclic triterpenoid of Lantana plants against acetaminophen-induced liver damage. Molecules. 2012, 17(12):13937-47.

Gumma SA., Enayat M., Hassan., and Khalifa DM. Hepatoprotective Effect of Aqueous Extracts of Some Medicinal Plant Mixtures on CCl_4-Induced Liver Toxicity. Journal of Pharmacy and Biological Sciences (IOSR-JPBS).2017, 12 (1): 43-52.

Gupta AK., Misra N. Hepatoprotective activity of aqueous ethanolic extract of *Chamomile capitula* in paracetamol intoxicated albino rats. Journal of Pharmacognosy and Phytochemistry Toxicology.2006, 1(1):17-20.

Gursoy N., Sarikurkcu C., Cengiz M., and Solak MH., Antioxidant activities, metal contents, total phenolics and flavonoids of seven Morchella species.Food Chem Toxicol. 2009, 47 (9):2381-88.

Gutierrez RM., Mitchell S., Solis RV., Psidium guajava: a review of its traditional uses. phytochemistry and pharmacology. J Ethnopharmacol. 2008: 117(1):1-27.

Guyton AC., Hall JE., In: Guyton AC and Hall JE, editors. Textbook of medical physiology. 11th ed. Philadelphia, PA: Elsevier Inc, 2006.

Gyawali R., Shrestha A., Khanal A., Pyakurel AS., Joshi N., Bajaj P., Chaudhary P., and Thapa R. Hepatoprotective properties of selected plants against paracetamol-induced hepatotoxicity in mice. Journal of Institute of Science and Technology. 2017, 22(1):147-151.

Hadriche O., Ammar S., Contreras DM., TurkiM., Carretero AS., and Feki EA. Antihyperlipidemic and antioxidant activities of edible tunisian *Ficuscarica* L. fruits in high fat diet-induced hyperlipidemic rats. Plant Foods Hum.Nutr.2016, 71:183–189.

Hasan RSM., Hossain MMD., Raushanara A., Mariam J., Mazumder EHMD., and Rahman S. DPPH free radical scavenging activity of some Bangladesh medicinal plants. Journal of Medicinal Plants Research. 3 (11): 875-879.

Heibatollah S., Reza NM., Izadpanah G., and Sohailla S. Hepatoprotective effect of *Cichorium intybus* on CCl_4 induced liver damage in rats. Afr J Biochem Res. 2008, 2: 141-44.

Heidelbaugh JJ., and Bruderly M. Cirrhosis and chronic liver failure: part I. Diagnosis and evaluation. American Family Physician.2006, 74(5): 756-762.

Hodgson E., and Levi PE., Hepatotoxicity. In: Hodgson E, editor. A textbook of modern toxicology. 3rd ed. Hoboken, New Jersey, USA: John Wiley & Sons, Inc.; 2004.

Hong and Francis. "History of medicines in China''Mc Gill Journal of Medicine.2004, 8(1): 79-84.

Hong CQ., Lee HA., Rhee CH., Choung SY., and Lee KW. Separation of the Antioxidant Compound Quercitrin from Lindera obtusiloba Blume and its Antimelanogenic Effect on B16F10 Melanoma Cells. Bioscience, Biotechnology, and Biochemistry.2013, 77 (1): 58-64.

Hsouna BA., SaoudiM., Trigui M., Jamoussi K., Boudawara T., Jaoua S., and EL Feki A. Characterization of bioactive compounds and ameliorative effects of Ceratonia siliqua leaf extract against CCl_4 induced hepatic oxidative damage and renal failure in rats. Food Chemical Toxicology.2011, 49: 3183–3191.

Hue MS., Boyce AN., and Somasundram C. Antioxidant activity, phenolic and flavonoid contents in the leaves of different varieties of sweet potato (*Ipomoea batatas*) Australian journal of crop sciencesAJCS.2012, 6(3):375-380.

Husain I., Bano A., and Ullah F. Traditional drugs therapies for various medicinal plants of central Karakorum National Park Gilgit Baltastan (Pakistan), Pakistan Journal of Botany. 2011, 43:79-84.

Hussain L., Akash MSH., Tahir T., Rehman K., and Ahmed KZ. Hepatoprotective effects of methanolic extract of *Alcea rosea* against acetaminophen-induced hepatotoxicity in mice Bangladesh J Pharmacol. 2014, 9: 322-327.

Hussain T., Fareed S., Siddiqui HH., Vijaykumar M., and Rao CV. Acute and subacute oral toxicity evaluation of Tephrosia purpurea extractin rodents, Asian Pacific Journal of Tropical Disease. 2012, 2 (2): 129-132.

Ibrahim M., Khaja NM., Aara A., Khan AA., Habeeb MA., and Devi PY. Hepatoprotective activity of *Sapindus mukorossi and Rheum emodi extracts*. World Journal of Gastroenterology. 2008, 14(16): 2566–2571.

Ihsan-Ul-Haq., Nazif Ullah., Bibi G., Kanwal S., Ahmad MS., and Mirza B. Antioxidant and Cytotoxic Activities and Phytochemical Analysis of *Euphorbia wallichii* Root Extract and its Fractions. Iranian Journal of Pharmaceutical Research. 2012, 11 (1): 241-249.

Ijaz S., Khan HMS., Anwar Z., Talbot B., and Walsh JJ. HPLC profiling of Mimosa pudica polyphenols and their non-invasive biophysical investigations for anti-dermatoheliotic and skin reinstating potential Biomedicine & Pharmacotherapy. 2019, 109: 865–875.

Ilyas U., Katare, DP., Aeri V., and Naseef, PP. A Review on Hepatoprotective and Immunomodulatory Herbal Plants. Pharmacognosy Reviews. 2016, 10(19): 66–70.

Ishtiaq M., Mahmood A., and Maqbool M. Indigenous knowledge of medicinal plants from Sudhanoti district (AJ&K), Pakistan Ethnopharmacol.2015, 20 (168):201-7. doi: 10.1016/j.jep.

Ismail M., Hussain J., Khan A., Khan LA., Ali L., Farman-ullah K., Amar Zada K., and Niazandin JU. Antibactrial, antifungal, cytotoxic, phytotoxic, insecticidal and enzyme inhibitory activities of *Geranium wallichianum*. Evidence Based complementary and alternative medicines. 2012, 1-8.

Ismail M., Ibrar M., Zafar IZ., Hussain H., Ahmad M., Ejaz A., and Chudhary IA. Chemical constituents and antioxidant activity of *Geranium wallichianum*. Records of Natural Products.2009, 3(4):193-197.

Itoh A., Isoda K., Kondoh M., Kawase M., Kobayashi M., Tamesada M., and Yagi K. Hepatoprotective Effect of Syringic Acid and Vanillic Acid on Concanavalin A-Induced Liver Injury.Biol. Pharm. Bull. 2009, 32 (7):1215—1219.

Izzreen MNQ., and Fadzelly ABM. "Phytochemicals and antioxidant properties of different parts of Camellia sinensis leaves from Sabah Tea plantation in Sabah, Malaysia." International Food Research Journal.2013, 20 (1): 307–312.

Jacob JM., Olaleye TM., and Olugbuyiro OAJ. Hepatoprotective effect of *Alchornea Cordifolia* leaves on liver damage in albino rats, International Journal of Applied Science and Biotechnology. 2014, 2(2): 217-221.

Jain A., Soni M., Deb L., JainA., Rout SP., Gupta VB., and Krishna KL. Antioxidant and hepatoprotective activity of ethanolic and aqueous extracts of *Momordica dioica* Roxb. leaves. Journal of Ethnopharmacology.2008, 115: 61–66.

Jain V., Murugananthan G., Deepak M., Viswanatha GL., and Manohar D. Isolation and Standardization of Various Phytochemical Constituents from Methanolic Extracts of Fruit Rinds of *Punica granatum* Chinese Journal of Natural Medicines.2011, 9 (6): 0414–0420.

Jalil MA., Rahman SM., Rahman A., Ridwan B., Rashid., and Rashid MA. Active Fractions from *Asparagus racemosus* Willd. With Thrombolytic, Membrane Stabilizing and Free Radical Scavenging Activities.Bangladesh Pharmaceutical Journal.2015, 18(2): 183-186.

Jumuna S., Paulsamy S., and Karthika K. Screening of *in vitro* antioxidant activity of methanolic leaf and root extracts of *Hypochaeris radicata* L. (Asteraceae). Journal of Applied Pharmaceutical Science.2012, 02 (07): 149-154.

Janbaz K., Saeed S., and Gilani A. Studies on the protective effects of caffeic acid and quercetin on chemical-induced hepatotoxicity in rodents. Phytomedicine. 2004, 11: 424-30.

Kale I., Khan MA., Irfan Y., and Goud VA. Hepatoprotective potential of ethanolic and aqueous extract of flowers of Sesbania grandiflora (Linn) induced by CCl_4. Asian Pac J Trop Biomed. 2012: 2(2): S670-S679.

Kashawa V., Neema A., and Agarwal A. Hepatoprotective prospective of herbal drugs and their vesicular carrier, a review. International Journal of Research in Pharmaceutical and Biomedical Sciences.2011, 2(2): 360-374.

Khaleefa A., Irshad A., Nawchor., Bhat AM., Aijaz HG., and Nadia A. Ethnomorphological review of genus Primula. International Journal of Advance Research.2014, 2 (4): 29-34.

Khan H., Saeed M., Khan M., Khan I., Ahmad M., Muhammad N., and Khan A. Antimalarial and free radical scavenging activities of rhizomes of *Polygonatum verticillatum* supported by isolated metabolites. Med Chem Res. 2012, 21:1278–1282.

Khan MA., Ajab M., and Husain M. Medicinal plants used in folk recopies by inhabitants of Himalayan region Poonch valley Azad Kashmir (Pakistan). Journal of Basic and Applied Sciences. 2012, 8: 35-45.

Khan MA., Husain M., and Mujtaba G. An Ethno-botanical inventory of Himalayan region Poonch valley Azad Kashmir (Pakistan). Ethno-botany Research and Application. 2010, 8 (1): 107-123.

Kim EY., Kim EK., Lee HS., Sohn Y., SohY., Jung HS., and Sohn NW. Protective effects of Cuscutae semen against dimethylnitrosamine-induced acute liver injury in Sprague-Dawley rats. Biol Pharm Bull. 2008,30(8): 1427-1431.

Kiran PM., RajuA., Rao BG. Investigation of hepatoprotective activity of Cyathea gigantea (Wall. ex. Hook.) leaves against paracetamol-induced hepatotoxicity in rats. Asian Pac J Trop Biomed. 2012, 2(5): 352-356.

Kobakhidze KB., and Alaniya MD. Flavonoids from *Geranium robertianum*. Chem. Nat. Compd.2004, 40: 89–90.

Kokhdan EP., Ahmadi K., Sadeghi H., Sadeghi H., Dadgary F., Danaei N., and Aghamaali MR. Hepatoprotective effect of *Stachys pilifera* ethanol extract in carbon tetrachloride-induce hepatotoxicity in rats. Pharmaceutical Biology. 2017, 55(1): 1389–1393.

Komalavalli T., Lincy PM., Muthukumarasamy S., and Mohan VR. Anticancer activity of ethanol extract of *Asytasia travancorica* Bedd (Acanthaceae) whole plant against dalton ascites lymphoma. Journal of Advanced Pharmacy Education and Research. 2014, 4 (2): 113-118.

Kothandaraman G., and Shanmugam S., Phytochemical estimation and *in vitro* antioxidant activity of rhizome of *in vitro* regenerated *Zingiber zerumbet* (L.) Sm. Journal of Pharmacognosy and Phytochemistry. 2018, 7(5): 566-570.

Krishna KI., Mruthunjaya K., and Patel JA. Antioxidant and Hepatoprotective Potential of stem Methanolic Extracts of *Juticia gendarussa* Burn. Int. J. Pharm. 2010, 6(2):72-80.

Kshirsagar A., Ingawale D., Ashok P., and Vyawahare N. Silymarin: A comprehensive review. Pharmacognosy Reviews.2009, 3(5), 116-124.

Kukula-Koch W. "The Elevation of LC-ESI-Q-TOF-MS Response in the Analysis of IsoquinolineAlkaloids from Some Papaveraceae and Berberidaceae Representatives." Journal of Analytical Methods in Chemistry. 2017,1-9. Article ID 8384107, https://doi.org /10.1155/2017/8384107.

Kulisic T., Radonic A., Katalinic V., and Milos M. Use of different methods for testing antioxidative activity of oregano essential oil. *Food Chem*. 2004, 85: 633-640.

Kumar A., Rai N., Kumar N., Gautam P., and Kumar JS. Mechanisms involved in hepatoprotection of different herbal products, a review. Int J Res Pharm Sci. 2013, 4 (2):112-7.

Kumar R and Gnanadesigan M., Hepatoprotective and Antioxidant Properties of Rhizophora mucronata plant in CCl_4 Intoxicated Rats. J Exp Clin Med. 2012, 4: 66-72.

Kumar S and Pandey AK. Chemistry and Biological Activities of Flavonoids: An Overview. Hindawi Publishing Corporation. The Scientific World Journal. 2013, 1-16. Article ID 162750.

Kumaresan R., Veerakumar SV., Elango. A Study on Hepatoprotective Activity of *Mimosa pudica* in Albino Rats. International Journal of Pharmacognosy and Phytochemical Research. 2015, 7 (2): 337-339.

Kvasnicka F., Bíba B., Sevcík R., Voldrich M., and Krátká J. Analysis of the active components of silymarin. Journal of Chromatography A.2003, 990 (1): 239-245.

Laouar A., Klibet F., Bourogaa E., Benamara A., Boumendjel A., Chefrour A., and Messarah M. Potential antioxidant properties and hepatoprotective effects of *Juniperus phoenicea* berries against CCl_4 induced hepatic damage in rats. Asian Pacific Journal of Tropical Medicine. 2017, 10(3): 263–269.

Laszczyk M., Jager S., Simon-Haarhaus S., Scheffler A., and Schempp CM. Physical, chemical and pharmacological characterization of a new oleogel-forming triterpene extract from the outer bark of birch (betulae cortex). Planta Med.2006, 72:1389-1395.

Law K., and Brunt EM., Nonalcoholic fatty liver disease. Clinics Liver Dis. 2010, 14(4): 591-604.

Lee JA., Uhlik MT., Maxhom CM., Tomandal., Sall D., and DJ. Modern phenotypic drugs discovery is available, neoclassic pharma strategy, Journal of Medical Chemistry.2012, 55 (10): 4527-4538.

Levy C., Seeff LD., and Lindor KD. Use of herbal supplements for chronic liver disease. Clin Gastroenterol Hepatol. 2004, 2: 947-956.

Liao RC., Chang SY., Peg HW., Lai CH., and Ho LY. Analgesic and anti-inflammatory activities of methanolic extract of *elaeagnus oldhamii* maxim in mice. American Journal of Chinese Medicine.2012, 40 (3): 581-597.

Lieber CS. Role of oxidative stress and antioxidant therapy in alcoholic and nonalcoholic liver diseases. Advances in Pharmacology.1996, 38: 601-628.

Liu SC., Lin JT., Wang CK., *et al.* Antioxidant properties of various solvent extracts from lychee (Litchi chinenesis sonn.) flowers. Food Chem. 2009, 114:577-81.

Liu W., Yin D., Wang DM., and Li DW. Influence of environmental factors on the contents of active ingredients and radical scavenging property of potentilla fruticosa in the main production areas of China. Pak. J. Bot. 2015, 47(6): 2195-2205.

Longo LD. Harrison's principles of internal medicine (18th edition) New York: Mc Graw-Hill. P. liver Transplantation.2011, ISBN 9780071748896.

Magama S., Lieta MI., and Asita AO. Antioxidant and free radical scavenging properties of four plant species used in traditional medicine in Lesotho. International Journal of Medicinal Plant Research. 2013, 2 (3): 170-178.

Malik F., Mirza T., Riaz H., Hameed A., and Hussain S. Biological screening of seventeen medicinal plants used in the traditional systems of medicine in pakistan for antimicrobial activities. African Journal of Pharmacy and Pharmacology.2010, 4(6): 335-340.

Malyshev LI., and Peshkova GA., Flora Tsentralnoy Sibiri, Nauka, Novosibirsk, 1979, 1: 276-292.

Manjunath BK., and Vidya SM. Hepatoprotective activity of Vitex trifolia against carbon tetrachloride-induced hepatic damage. Indian Journal of Pharmacology. 2008, 7(2): 241-245.

Marcellin P., Blaise K., and Kutala. Liver diseases: A major, neglected global public health problem requiring urgent actions and large-scale screening, *Liver International*.2018, 38 (1): 2–6.

Maria M., Arshad M., Asif S., and Chaudhry KS. *Viola canesences*: Herbal wealth to be conserved, Hindawi Publishing Corporation. Journal of Botany.2014, 1- 7.

Mathur A., Satish K., Verma S., Santosh K., Singh G.B.K.S., and Prasad VK. Investigation of the Antimicrobial, Antioxidant and Anti-inflammatory Activity of Compound Isolated from Murraya Koenigii. International Journal of Applied Biology and Pharmaceutical Technology.2011, 2: 470-475.

Matic S., Stanić S., Bogojević D., Vidaković M., Grdović N., Dinić S., and Mihailović M. Methanol extract from the stem of *Cotinuscoggygria* Scop., and its major bioactive phytochemical constituent myricetin modulate pyrogallol-induced DNA damage and liver injury. Mutat Res-Gen Tox En. 2013, 755: 81-89.

Mattos GTC., Maurício ÂQ., Rettori D., Alonso A., and Lima HM. Antioxidant Activity of Caffeic Acid against Iron-Induced Free Radical Generation—A Chemical Approach. PLoS ONE. 2015,10 (6):1-12.

Mekky RH., Fayed MR., El-Gindi MR., Abdel-Monem AR., Contreras MdM., Segura-Carretero A., and Abdel-Sattar E. Hepatoprotective Effect and Chemical Assessment of a Selected Egyptian Chickpea Cultivar. Front. Pharmacol. 2016, 7: 1-9. Article 344.

Mhalla D., Zouari K., Bouassida., Chawech R., Bouaziz A., Makni S., Jlaiel J., Tounsi S., Jarraya MZ., and Trigui M. Antioxidant, Hepatoprotective, and Antidepression Effects of *Rumex tingitanus* Extracts and Identification of a Novel Bioactive Compound. Hindawi Bio Med Research International. 2018, ArticleID,7295848, 10 pages.

Mindikoglu AL., Regev A., and Schiff ER. Hepatitis B reactivation after cytotoxic chemotherapy: the disease and its prevention. Clinical Gastroenterology Hepatology. 2006, 4: 1076-81.

Mir A., Anjum F., Riaz, N., Iqbal, H., Wahedi, H.M., Khattak, J.Z.K., Khan, M.A., and Malik SA. Carbon tetrachloride (CCl_4) - induced hepatotoxicity in rats: Curative role of Solanum nigrum. Journal of Medicinal Plants Research. 2010, 4 (23): 2525-2532.

Mistry S., Dutt KR., and Jena J. Protective effect of Sida cordata leaf extract against CCl_4 induced acute liver toxicity in rats. Asian Pac J Trop Med. 2013, 6(4): 280-284.

More AN., Pradeep B., Parab., and Aptle KG. Evaluation of activity of whole stem extracts of *Oroxylum indicum* on paracetamol induced hepatotoxicity. Int J Pharm Bio Sci. 2013, 4 (4): 255 – 265.

Morenoa MG., and Reyes GG. The role of oxidative stress in the development of alcoholic liver disease Revista de Gastroenterología de México. 2014, 79(2):135-144.

Mosquera OM., Correa YM., Buitrago DC., and Niño J. "Antioxidant activity of twenty-five plants from Colombian biodiversity." Memorias do Instituto Oswaldo Cruz. 2007, 102 (5): 631–634.

Moyo M., Amoo SO., Ncube B., Ndhlala AR., Finnie JF., Staden JV. Phytochemical and antioxidant properties of unconventional leafy vegetables consumed in southern Africa. S. Afr. J. Bot.2013, 84:65-71.

Mroueh M., Saab Y., and Rizkallah R. Hepatoprotective activity of *Centaurium erythraea* on acetaminophen-induced hepatotoxicity in rats. Phytother Res. 2004, 18:431-3.

Mukazayire., AllaeysV., Calderon B.P., Vigny S.C., Bigendako JM., and Duez P. Evaluation of the hepatotoxic and hepatoprotective effect of Rwandese herbal drugs on *in vivo* (guinea pigs' barbiturate-induced sleeping time) and *in vitro* (rat precision-cut liver slices, PCLS) Models. Experimental and Toxicologic Pathology.2010, 62: 289–299.

Murugan R., and ParimelazhaganT. Comparative evaluation of different extraction methods for antioxidant and anti-inflammatory properties from Osbeckia parvifolia Arn. – An in vitro approachJournal of King Saud University – Science. 2014, 26: 267–275.

Nada SA., Omara EA., Abdel-Salam OM., and Zahran HG. Mushroom insoluble polysaccharides prevent carbon tetrachloride-induced hepatotoxicity in rat. Food Chem Toxicol. 2010, 48 (11): 3184-8.

Naeem A., Mettu P., Obando MLS., and Martin A. Deferasirox induced liver injury in haemochromatosis, Journal of The College of Physicians and Surgeons Pakistan.2010, 20 (8): 551-553.

Nagpal M., and Sood S. Role of curcumin in systemic and oral health: an overview. J Nat Sci Biol Med. 2013, 4(1) :3-7.

Najmus-saqib Q., Alam F., and Ahmad M. Antimicrobial and cytotoxicity activities of medicinal plant *Primula macrophylla*, Journal of Enzyme Inhibition Medical Chemistry.2009, 24(3): 697-701.

Natarajan SK., ThomasS., RamamoorthyP., Basivireddy J., Pulimood AB., Ramachandran, A., and Balasubramanian KA. Oxidative stress in the development of liver cirrhosis: a comparison of two different experimental models. Journal of Gastroenterology and Hepatology.2006, 21(6): 947-957.

Nazir N., Zahoor M., Nisar M., Khan I., Karim N., Abdel-Halim H., and Ali A. Phytochemical analysis and antidiabetic potential of *Elaeagnus umbellata* (Thunb.) in streptozotocin-induced diabetic rats: pharmacological and computational approach, BMC Complementary and Alternative Medicine. 2018, 18:332.https://doi.org/10.1186/s12906-018-2381-88.

Neagu E., Paun G., Constantin, D., Radu GL. Cytostatic activity of *Geranium robertianum* L. extracts processed by membrane procedures. Arabian J. Chem. 2013, 10: S2547-S2553.

Newman DJ., Cragg GM., and Snader KM. The influence of natural products upon drug discovery. Nat. Prod. Rep. 2000,17: 215–234.

Nguyen TY., To DC., Tran MH., Lee JS., Lee JH., and Kim JA., *et al.* Anti-inflammatory flavonoids isolated from Passiflora foetida. Nat Prod Comm. 2015: 10: 929–31.

AbramovicH., Abram V., Čuk A., Čeh B., Možina SM., Vidmar M., Pavlovič M., and Ulrih P, Antioxidative and antibacterial properties of organically grown thyme (Thymus sp.) and basil (Ocimum basilicum L.). Turk J Agric For. 2018, 42: 185-194.

Nithianantham K., Shyamala M., ChenY., Latha LY., Subramanion L., Jothy., and Sasidharan S. Hepatoprotective Potential of *Clitoria ternatea* Leaf ExtractAgainst Paracetamol Induced Damage in Mice. Molecule.2011, 16:10134-10145.

Nithya N., and Balakrishnan PK. Evaluation of some medicinal plants for their antioxidant properties. International Journal of Pharma Tech Research. 2011, 3(1): 381-385.

Njoku VO., and Obi C. Phytochemical constituents of some selected medicinal plants. African Journal of Pure and Applied Chemistry.2009, 3(11): 228-233.

Nooman A., Khalaf., Ashok K., Shakya., Al-othman A., El-agbar z., and Farah H. Antioxidant Activity of Some Common Plants.Turk J Biol.2008, 32: 51-55.

OECD. OECD Guideline for testing of chemicals. Acute Oral Toxicity—Acute Toxic Class Method, Guideline No. 423. Adopted December 2001. Organisation for Economic Cooperation and Development, Rome, Italy.

Okazaki I., Noro T., Tsutsui., Yamanouchi E N., Kuroda H., Nakano M., Yokomori H., and Inagaki. Fibrogenesis and Carcinogenesis in Nonalcoholic Steatohepatitis (NASH): Involvement of Matrix Metalloproteinases (MMPs) and Tissue Inhibitors of Metalloproteinase (TIMPs). Cancers (Basel). 2014, 6 (3): 1220-1255.

Okazaki I., Watanabe T., Hozawa S., Niioka M., Arai M., and Maruyama K. Reversibility of hepatic fibrosis: from the first report of collagenase in the liver to the possibility of gene therapy for recovery.Keio J Med. 2001, 50(2): 58-65.

Orhan EI., Murat K., Asakar AM., Serez SF., Gulderen Y., and Bilgs. Free radical scavenging properties and phenolic characterization of some edible plants. Food Chemistry.2009, 114 (1): 276-281.

Osadebe PO., Okoye FB., Uzor PF., Nnamani NR., and Adiele IE., *et al.* Phytochemical analysis, hepatoprotective and antioxidant activity of *Alchornea cordifolia* methanol leaf extract on carbon tetrachloride induced hepatic damage in rats. Asian Pacific J Trip Med.2012, 5: 289-93.

Oszmianski J., Nowicka P., Teleszko.M., Cebulak AWT., and Oklejewicz K. Analysis of Phenolic Compounds and Antioxidant Activity in Wild Blackberry Fruits, *Int. J. Mol. Sci.*2015,16 (7): 14540–14553.

Oszmianski JJ., Kolniak-Ostek A., and Wojdyło.Application of ultra-performance liquid chromatography photodiodedetector-quadrupole/time of flight-spectrometry (UPLC-PDA-Q/TOF-MS) method for the characterizationof phenolic compounds of *Lepidium sativum* L. sprouts. Eur Food Res Technol.2013, 236:699–706.

Oyinloye BE., Ajiboye BO., Ojo OA., Musa HM., Onikanni SA. , and Ojo AD. Ameliorative potential of *Aframomum melegueta* extract in cadmiuminduced hepatic damage and oxidative stress in male Wistar rats. Journal of Applied Pharmaceutical Science. 2016, 6 (07): 094-099.

Paganga G., Miller N., and Rice-Evans CA. The polyphenolic content of fruit and vegetables and their antioxidant activities. What does a serving constitute, Free Radical Res.1999, 30: 153-162.

Pandey G. Medicinal plants against liver diseases, International Research Journal of Pharmacy.2012, 2(5): 115-121.

Pandian MR, Banu GS, and Kumar G. A study of antimicrobial activity of Alangium salviifolium. Indian J Pharmacol. 2006, 38: 203-204.

Park EJ., Jeon CH., KoG., Kim J., and Sohn DH. Protective effect of curcumin in rat liver injury induced by carbon tetrachloride. J. Pharm. Pharmacol.2000, 52: 437-440.

Park YS., Jung ST., Kang SG., Heo BG., Arancibia-Avila P., and Toledo F.*et al.* Antioxidants and proteins in ethylene- treated kiwifruits. Food Chem.2008, 107: 640-648.

Parma SR., Vashrambhai PH., and Kalia K. Hepatoprotective activity of some plants extract against paracetamol induced hepatotoxicity in rats. Journal of Herbal Medicine and Toxicology. 2010, 4 (2): 101-106.

Patel JA., and Shah US. Hepatoprotective activity of Piper longum traditional milk extract on carbon tetrachloride induced liver toxicity in wistar rats. Bulletin Latino American aromatics.2009: 8(2):121-128.

Patil AP., and Patil VR. *Clitoria ternatea* Linn. An overview. International Journal Pharmaceutical Research. 2011, 3: 20-23.

Peschel W., Dieckmann W., Sonnenschein M., and Plescher A. High antioxidant potential of pressing residues from evening primrose in comparison to other oilseed cakes and plant antioxidants. Industrial Crops and Products. 2007, 25:44–54.

Piana M., Zadra M., Brum TF., Boligon AA., Gonçalves AF., and Dacruz RC., *et al.* Analysis of rutin in the extract and gel of Viola tricolor. J Chromatogr Sci.2013, 51: 406-411.

Pingale SS. Hepatoprotection study of leaves powder of *Azadrachta indica* and *A. Juss*. International Journal of Pharmaceutical Sciences Review and Research.2010, 3: 37-42.

Ponmari G., Annamalai A., Gopalakrishnan VK., Lakshmi PTV., Guruvayoorappan C. NF-kB activation and proinflammatory cytokines mediated protective effect of *Indigofera caerulea* Roxb. On CCl_4 induced liver damage in rats. Int Immunopharm. 2014, 23(2): 672-680.

Pradhan SC., and Girish C. Hepatoprotective herbal drug, silymarin from experimental pharmacology to clinical medicine.Review. Indian J Med Res. 2006, 124(5):491-504.

Preethi JK., Vennila S., Penislusshiyan S., and Velvizhi. Hepatoprotective and Antioxidant Role of *Ziziphus jujube* Leaves on Paracetamol Induced Hepatic Damage in Rats Journal of Diseases and Medicinal Plants2016, 2(1-1): 1-10.

Prochazkova DI., Bousova., and Wilhelmova N. Antioxidant and prooxidant properties of flavonoids. Fitoterapia.2011, 82(4): 513–523.

Purushottam B., Rakhunde., and Ali SA. Antioxidant and Cytoprotective effect of *Fragaria nubicola* on ischemiareperfusion induced brain injury. Annals of Experimental Biology.2014,2 (4):33-38.

Qaiser M. Polygonaceae. In: Ali, S. I. and Qaisar, M. (Eds.). Flora of Pakistan. Department of Botany, Karachi University and Missouri Botanical Garden, St. Louis, Missouri, U.S.A. 2001, 205: 4-164.

Qamar QZ., Anwar M., Dar NI., and Ali U. Ethno-botanical study of wild medicinal plants of Neelum Valley, Azad Jammu and Kashmir, Pakistan. Pak J Wild. 2010, 1(1): 25-30.

Qureshi AR., Ghufran AM., Gilani AS., Sultana K., and Ashraf M. Ethno-botanical studies of selected medicinal plants of sudhan gali and Ganga Chotti hills Bagh, Azad Kashmir(Pakistan). Pakistan Journal of Botany.2007, 39(7): 2275-2283.

Rafique MK., Rada AS., Arshad M., Rashid A., and Razaq A. Antioxidant potential of indigeneous medicinal plants of district Gujrat Pakistan. Journal of Pharma Science Tech.2014, 4(1): 21-22.

Rahim, ZHA., and Khan HBSG. Comparative studies on the effect of crude aqueous (CA) and solvent (CM) extracts of clove on the cariogenic properties of Streptococcus mutans. Journal of Oral Science.2006, 48(3): 117-123.

Rahman S., Awan RZ., Khan A., Shah SA., Rahman A., and Gul I. Antibacterial potential of *skemmia laureola* methonolic extract. Global Advanced Research Journal of Microbiology.2012, 1(6): 090-093.

Rajesh MG., and Latha MS. Preliminary evaluation of the antihepatotoxic activity of Kamilari, a polyherbal formulation. J. Ethnopharmaco.2004,91:99-104.

Raju RW., Radhika SS., Kunal MT., Kalpana SP., and Sunil SJ. Int J Green Pharm. 2008, 2: 220- 223.

Ramadori G., Moriconi F. Malik I., and Duadas J. Physiology and pathophysiology of liver inflammation, damages and repair. Journal of Physiology and Pharmacology.2008,1: 107-117.

Rao CV., Rawat AK., Singh AP., Singh A., and Verma N. Hepatoprotective potential of ethanolic extract of *Ziziphus oenoplia* (L.) Mill roots against antitubercular drugs induced hepatotoxicity in experimental models. Asian Pac J Trop Med. 2012, 5(4):283-8.

Rashed KN., Chang CW., Wu LY., and Peng WH. Hepatoprotective activity of *Diospyros lotus* fruits on acute liver injury induced by carbon tetrachloride and phytochemical analysis. Topcls J Herb Med. 2013, 2: 75-83.

Rawat AK., Mehrotra S., Tripathi SC., Shome U. Hepatoprotective activity of *Boerhaavia diffusa* L. roots a popular Indian ethnomedicine. J Ethno Pharmcol.1997, 56: 61-66.

Raza SS., Khan MM., Ashafaq M., Ahmad A., Khuwaja G., and Khan A., *et al.* Silymarin protects neurons from oxidative stress associated damages in focal cerebral ischemia:

a behavioral, biochemical and immunohistological study in Wistar rats. J Neurol Sci. 2011, 309(1-2): 45-54.

Rebecca J., and Robbins. Phenolic acids in foods: An overview of analytical methodology. J. Agric. Food Chem. 2003, 51: 2866-2887.

Reddy VR., Kumar V., and Reddy MK. Hepatoprotective Activity of *Elytraria acaulis* plant extracts in Albino Rats. International Journal of Pharmacognosy and Phytochemical Research. 2014, 15 6(4): 925-929.

Rehan T., Tahira R., RehanT., BibiA., and Naeemullah M. Screening of seven medicinal plants of family lamiaceae for total phenolics, flavonoids and antioxidant activity. Pakhtunkhwa Journal of Life Science.2014, 2: 107-117.

Rehm J., Andriy V., Samokhvalov., Kevin D., Shield. Global burden of alcoholic liver diseases. Journal of Hepatology. 2013, 59:160–168.

Rehman H., Begum W., Anjum F., and Tabasum H. *Rheum emodi* (Rhubarb) A fascinating herb. Journal of Pharmacognosy and Phytochemistry.2014, 3 (2): 89-94.

Rehman K., Akash.,MSH ., AzharS., KhanSA ., AbidR., Waseem A., Murtaza G., and Sherazi. TA. A Biochemical and Histopathologic Study Showing Protection and Treatment of Gentamicin-Induced Nephrotoxicity in Rabbits Using Vitamin C. Afr J Tradit Complement Altern Med. 2012, 9(3): 360–365.

RejaieASS., Aleisa AM., Sayed-Ahmed MM., Al-Shabanah OA., Abuohashish HM., and Ahmed MM., *et al.* Protective effect of rutin on the antioxidant genes expression in hypercholestrolemic male Westar rat. BMC Complement Altern Med. 2013, 13: 136-145.

Ritesh KR., Suganya A., DileepkumarY., RajashekarT., Shivanandappa. A single acute hepatotoxic dose of CCl_4 causes oxidative stress in the rat brain.Toxicology Reports. 2015, 2: 891-895.

Robson B., and Vaithiligam A. Drug gold and data dragons, myths and realities of data mining in the pharmaceutical industry, in pharmaceutical data mining: Approaches and

Applications for Drug Discovery, John Wiley & Sons, Inc., Hoboken, NJ, USA. 2009, doi: 10.1002/9780470567623.ch2.

Russmann S., Kullak-Ublick GA., and Grattagliano I. Current concepts of mechanisms in drug-induced hepatotoxicity. Chemistry.2009, 16(23): 3041-3053.

Ruth B. Medicinal properties of *Litsea glutinosa* Lour. and *Litsea monopetala*. e-Planet. 2004, 2: 94-95.

Saba AB., Onakoya OM., Oyagbemi AA. Hepatoprotective and in vivo antioxidant activities of ethanolic extract of whole fruit of Lagenaria breviflora. J Basic Clin Physiol Pharmacol. 2012: 23 (1):27-32.

Sabir SM., Rocha JBT., Water-extractable phytochemicals from *Phyllanthus niruri*exhibit distinct in vitro antioxidant and in vivo hepatoprotective activity against paracetamol-induced liverdamage in mice. Food Chem. 2008, 111: 845–851.

Saleem M., Karim A., Ahmad M.S., Qadir I., and Husain S.N. Hepatoprotective effect of aqeous methanolic extract of *Rumexdentatus* in paracetamol-induced hepatotoxicity in mice. Bangladesh Journal of Pharmacology.2014, 9: 284-289.

Sallie R., JM., Tredger and William R., Drugs and the liver. Part I. Testing liver function. Biopharm Drug Disp. 1991,12: 251-259.

Sana M., Bhatti A.S., and Shazad A.W. Protective effects of silymarin in isoniazid induced hepatotoxicity in rabbits, Annals of King Edward Medical University. 2010, 16 (1): 43-47

Saoudi M., and El Feki A. Protective role of Ficus carica stem extract against hepatic oxidative damage induced by methanol in male Wistar rats. Evid Based Complement Alternat Med. 2012,150458. Available from: http://dx.doi.org/doi:10.1155/2012/150458.

Sapakal VD., Ghadge RV., Adnaik RS., Naikwade AS., Magdum CS. Comparative hepatoprotective activity of Liv-52 and livomyn against carbon tetrachloride induced hepatic injury in rats. Int. J. Green. Pharm. 2008, 2:79-82.

Sayed AE., Martiskainen O., Din SHS., Abdel-Nasser A., Sabra., Olfat A., Hammam. Naglaa M., El-Lakkany M., and Abdel DM. Hepatoprotective and antioxidant effect of *bauhinia hookeri* extract against carbon tetrachloride induced hepatotoxicity in mice and characterization of its bioactive compounds by HPLC-PDA-ESI-MS/MS, Hindawi Publishing Corporation. Bio Medical Research. 2014, 9-14. Article ID 245171.

Scalbert A., and Williamson G. Dietary intake and bioavailablity of polyphenols. J Nutr. 2000, 130 (8S Suppl): 2073S-85S. doi: 10.1093/jn/130.8.2073S.

Schuppan S., and Afdhal, NH. Liver cirrhosis. The Lancet.2008, 371(9615): 838-851.

Serairi-Beji R., Wannes WA., Hamdi A., Ksouri RTR., Saidani-Tounsi M., and Bouraoui K MLN. Antioxidant and hepatoprotective effects of *Asparagus albus* leaves in carbon tetrachloride-induced liver injury rats. J. Food Biochem. 2018, 42 (1):12433. https://doi.org/10.1111/jfbc. 12433.

Seto WK., and Yuen MF. Non-alcoholic fatty liver disease in Asia: emerging perspectives.J Gastroenterol. 2017, 52 (2):164-174.

Shah L., Hong M., Tan HY., Wang N., and Feng Y. Insights into the Role and Interdependence of Oxidative Stress and Inflammation in Liver Diseases. Hindawi Publishing Corporation Oxidative Medicine and Cellular Longevity. 2016, 1-21. Article ID 4234061.

Shah LHY., Tan N., and Wang. "The role of oxidative stress and antioxidants in liver diseases." International Journal of Molecular Sciences. 2015, 16 (11): 26087–26124.

Shah SMM., Sadiq A., Shah SMH., and Khan S. Extraction of saponins and toxicological profile of Teucrium stocksianum boiss extracts collected from District Swat, Pakistan. Biological Research. 2014, 47:65.

Shah ZN., Muhammad N., Azeem S., and Rauf A. Preliminary phytochemical and anti-radical profile of *Conyza sumatrensis*. Middle East Journal of Medicinal Plants Research.2012, 1(1): 05-08.

Shaheen H. Species composition, structural diversity, anthropogenic pressure and

sustainability of western himalayan forests of Bagh district, Azad Jammu and Kashmir, Pakistan, Ph.D. Thesis. 2010.

Shaheen H., Nazir J., Firdous S., and Khalid A. Cosmetic ethnobotany practiced by tribal women of Kashmir Himalayas. Avicenna Journal Phytomedicines.2014, 4 (4): 239-250.

Shaheen H., Qaseem MF., Amjad MS., and Bruschi P. Exploration of ethno-medicinal knowledge among rural communities of Pearl Valley; Rawalakot, District Poonch Azad Jammu and Kashmir. PLOS ONE. 2017, 12(9): 1-32.

Shaker E., and Mahmoud H., and Mnaa S. Silymarin, the antioxidant component and Silybum marianum extracts prevent liver damage. Food Chem Toxicol. 2010, 48(3):803-6.

Sharma SK., Arogya SM., Bhaskarmurthy DH., Agarwal A., and Velusami CC. Hepatoprotective activity of the *Phyllanthus* species on *tert*-butyl hydroperoxide (*t*-BH)-induced cytotoxi-city in HepG2 cells. Pharmacogn Mag. 2011, 7: 229-33.

Shehab NG., Gharbieh EA., Fatehia A., and Bayoumi. Impact of phenolic composition on hepatoprotective and antioxidant effects of four desert medicinal plants. BMC Complementary and Alternative Medicine. 2015, 15: 401. https://doi.org/10.1186 /s 12906-015-0919-6.

Shi GF., An LJ., Jiang B., Guan S., and Bao YM. *Alpinia* protocatechuic acid protects against oxidative damage *in vitro* and reduces oxidative stress *in vivo*. Neurosci Lett. 2006, 403: 206-210.

Shinde M., Dhalwa., Potdar M., and Mahadik KR. Application of quality control principles to herbal drugs. Int. J. Phytomed. 2009, 1: 4–8.

Shinwari ZK. Medicinal plants research in Pakistan. Journal of Medicinal Plants Research. 2010, 4(3): 161-176.

Shuyi Y., Wei CW., Yin F., Jian., and Sun N. Theraputic applications of herbal medicines for cancer patients. Evidence-Based Complementary and Alternative Medicines. 2013,1- 15. Article ID 302426.

Singhal KG., and Gupta GD. Hepatoprotective and antioxidant activity of methanolic extract of flowers of *Nerium ole*ander*a*gainst CCl_4-induced liver injury in rats. Asian Pacific Journal of Tropical Medicine. 2012, 677-685.

SingletonVL., Orthofer R., and Ravento RML. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin–Ciocalteu reagent. Methods in Enzymology.1999, 299: 152–178.

Sjogren MH., Cheathan JG., Feldman M., Friedman LS., Brandt LJ., Sleisenger E., and Fordtran S. Gastrointestinal and Liver Disease.9th edition. Philadelphia, pa: Saundres Elsvier.2010, Chapter. 77.

Smith C., Marks DA., and Lieberman M., Liver metabolism. Marks' basic medical biochemistry: a clinical approach. 2nd ed. Mishawaka, IN, USA: Lippincott Williams & Wilkins publishing; 2005.

Song EK., Kim JH., Kim JS., Cho H., Nan JX., and Sohn DH. Hepatoprotective phenolic constituents of *Rhodiola sachalinensis* on tacrine-induced cytotoxicity in Hep G2 cells. Phytother Res. 2003, 17: 563-65.

Spiridon I., Bodirlau R., and Teaca G. Total phenolic content and antioxidant activity of plants used in traditional Romanian herbal medicine. Cent. Eur. J. Biol. 2011, 6(3): 388-396.

Stickel F., and Schuppan D. Herbal medicine in the treatment of liver diseases. Digestive and Liver Diseas.2007, 39(4): 293-304.

Stravitz RT., Lefkowitch J.H., Fontana R.J., Gershwin M.E., Leung P.S., and Sterling RK. Autoimmune acute liver failure proposed clinical and histological criteria. Hepatology.2011, 53: 517-526.

Sudipta D., Choudhury DM., Mandal CS., and Talukdar DS. Traditional knowledge of ethno-botanical hepatoprotective plants used by certain ethnic communities of Taripura State. Indian Journal of Fundamental and Applied Life Sciences ISSN. 2012, 2 (1): 84-97.

Suja SR., Latha PG., Pushpangadan P., and Rajasekharan S. Aphrodisiac property of Helminthostachys zeylanica in mice. Journal of Tropical Medicinal Plants.2002, 3: 191-195.

Tahir M., Inam-ul-haq., Malik N.N., Latif Z.s., Naveed K.A., Hassan M., and Malik S.A. Hepatoprotective potential of *Rheum emodi* on Carbon tetra chloride (CCl_4) induced hepatic damage. Annals Pakistan Institute of Medical Sciences.2008, 4(3): 152-155.

Talluri MR., Gummadi VP., and Battu GR. Chemical Composition and Hepatoprotective Activity of *Saponaria officinalis* on Paracetamol-induced Liver Toxicity in Rats. Pharmacog J. 2018, 10 (6):1196-201.

Tang X., GaoJ., Wang Y., FanYM., Xu LZ., Zhao XN., Xu Q., and Qian ZM. Effective protection of *Terminaliacatappa* L. leaves from damage induced by carbon tetrachloride in liver mito-chondria. J. Nutr. Biochem. 2006, 17: 177-182.

Tangjitman K., Wongsawad C., Kamwang K., Sukko T., and Trisanthi C. Ethno-medicinal plants used for digestive system disorders by the Karen of Northern Thailand, Journal of Ethno-biology and Ethnomedicine.2015, 11(27): 11-24.

Trans AC., and Assoc. Hepatitis A and B Superimposed Chronic Liver Disease: Vaccine Preventable Diseases. Transsaction of the American Clinical and Climatological Association. 2006, 117: 227-238.

Tsala DE., Penlab BVNN., Mendimi NJ., Kouamouo J., and Dimo T. Protective activity of the stem bark methanol extract of *Alafia multiflora* against carbon tetrachloride-induced hepatotoxicity in rats. Int. J. Pharm. Sci. Rev. Res.2010, 3:157-163.

Timbrell J. Principles of Biochemical Toxicology. Boca Raton: 2009. 4th.edn. CRC Press, https://doi.org/10.3109/9781420007084.

Uboh FE., Okon IE., and Ekong MB. Effect of aqueous extract of *Psidium guajava* leaveson liver enzymes, histological integrity and hematological indices in rats. Gastroenterol Res. 2010, 3(1):32-8.

Ullah F., Khan S., Afridi K.A., and Rahman S. Frequency of different causes of

cirrhosisliver in local population, Gomal Journal of Medical Sciences.2012, 10 (2): 178-181.

Ullah R., Mansour S., Alsaid., Abdelaaty A., Shahat., Naser AA., Abdullah A., Al-Mishari, Adnan M., and Akash Tariq. Antioxidant and Hepatoprotective Effectsof Methanolic Extracts of *Zilla spinosa* and *Hammada elegans* Against Carbon Tetrachloride induced Hepatotoxicity in rats. Open Chemistry. 2018, 16 (1): 133–140.

Vaishali M., Pai RV., Kedilaya HP., and Hedge S. Preliminary phytochemical screenig of members of lamiaceae: *Leucas linifolia*, *Coleus aromaticus* and *Postemon patchouli*. International Journal of Pharmaceutical Science and Research.2013, 21(1): 131-137.

Valiathan M. Healing plants. Cur Sci. 1998, 75:1122–1126.

Valko M., Rhodes C., Moncol J., Izakovic M., and Mazur M. Free radicals, metals and antioxidants in oxidative stress-induced cancer. Chemico-biological Interactions.2006, 160 (1): 1-40.

Vania C., Graca., Isabel CFR., Ferreira., Paulo F., and Santos. Phytochemical composition and biological activities of Geraniumrobertianum L.: A review, Industrial Crops and Products. 2016, 87: 363–378

Vankat G., Reddy R., Kumar V.R., Rama V., Reddy K.M., and Narsimha Y. Preliminary hepatoprotective activity of medicinal plant extract against carbon tetra chloride induced hepatotoxicity in albino rats. International Journal of Recent Scientific Research. 2015, 6 (7): 4946-4951.

Vemula R., Reddy GVR., Kumar R., and Reddy MK. Hepatoprotectivity activity of medicinal plant extracts on albino rats. world journal of pharmacy and pharmaceutical sciences.2016, 5(2): 1275-1284.

Verma N., and Khosa RL. Hepatoprotective activity of leaves of *Zanthoxylum armatum* D C in CCl_4 induced hepatotoxicity in rats, Indian Journal of Biochemistry and Biophysica.2010, 47: 124-127.

Vilas A., Arsul RO., Ganjiwale PG., and Yeole. Phytochemical and pharmacological standardization of polyherbal tablets for hepatoprotective activity against carbon tetrachloride induced hepatotoxicity, International Journal of Pharmaceutical Sciences and Drug Research. 2010, 2(4): 265-268.

Virginia D., Britto., Devi PP., Prasad VLB., Dhawan A., Vivek G., Mantri., and Prabhune S. Medicinal plant extracts used for blood sugar and obesity therapy shows excellent inhibition of invertase activity, synthesis of nano particles using the extract and its cytotoxic and genotoxic effects. International Journal of Life Science and Pharma Research.2012, 2(3): 61-74.

Vysochina GI., and Khramova EP. Component Composition of Flavonols and Their Content in *Aconogonon alpinum* (All.) Schur Growing in the Altay. Chemistry for Sustainable Development. 2010, 18: 477-483.

Wang J., Ji L., Liu H., and Wang Z. Study of the hepatotoxicity induced by *Dioscorea bulbifera* L. rhizome in mice. Bio Sci Trends. 2010, 4(2): 9-85.

Wang Z., Li Z., Ye Y., Xie L., and Li X. Oxidative Stress and Liver Cancer: Etiology and Therapeutic Targets. Review Article. Hindawi Publishing Corporation Oxidative Medicine and Cellular Longevity. 2016, 1-10. Article ID 7891574: http:// dx.doi.org /10.1155/2016/7891574.

WanYH., KyH., Keong S., Yeap., Rahim AR., Omar RA., Hol C., and Alitheen BN. Traditional practice, bioactivities and commercialization potential of *Elephantopus scaber* Linn. Journal of Medicinal Plants Research. 2009, 3(13): 1212-1221.

WenYi & Wang, Ying & Feng, And Yu-Qi. A simple and rapid method for simultaneous determination of benzoic and sorbic acids in food using in-tube solid-phase microextraction coupled with high-performance liquid chromatography. Analytical and bioanalytical chemistry.2007, 388. 1779-87.

Wojdylo A., Oszmianski J., Czemerys R., Antioxidant activity and phenolic compounds in 32 selected herbs. Food Chemistry. 2007, 105: 940–949.

World Health Organization. WHO guideline on the use of safety-engineered syringes for intramuscular, intradermal and subcutaneous injections in health-care settings. 2015, WHO/HIS/SDS/2015.5.

Yadav RNS., and Agarwala M. Phytochemical analysis of some medicinal plants. Journal of Phytology.2011, 3(12): 10-14.

Yam MF., Basir R., Asmawi MZ., Ismail Z. Antioxidant and hepatoprotective effects of *Orthosiphon stamineus* Benth standardized extract. Am J Chin Med.2007, 35: 117–128.

Yang J., Zhu D., Ju B., Jiang X., and Hu J. Hepatoprotective effects of *Gentianella turkestanerum* extracts on acute liver injury induced by carbon tetrachloride in mice. Am J Transl Res. 2017, 9(2): 569-579.

Yang JH., Lin HC., and Mau JL. Antioxidant properties of several commercial mushrooms. Food Chem.2002, 77:229–235.

Yang L., Wang CZ., Ye JZ., and Li HT. Hepatoprotective effects of polyprenols from Ginkgo biloba L. leaves on CCl_4-induced hepatotoxicity in rats. Fitoterapia. 2011: 82(6): 834-840.

Yang SS. Alcoholic Liver Disease in the Asian Pacific Region with High Prevalence of Chronic Viral Hepatitis. Journal of medicinal ultrasound.2016, 24: 93-100.

Yang SY, Hong CO, Lee GP, Kim CT, Lee KW. The hepatoprotection of caffeic acid and rosmarinic acid, major compounds of Perilla frutescens, against t-BHP-induced oxidative liver damage. Food Chem Toxicol. 2013, 55:92–9.

Yao H., QiaoYJ., Zhao YL., Tao XF., Xu LN., Yin LH., QiY., and Peng JY. Herbal medicines and nonalcoholic fatty liver disease. World Journal of Gastroenterology.2016,**22** (30): 6890-6905.

Yash RC. Salmonaella: Toxicity in rabbit ileum and liver by light and electron microscopy. Indian Journal of Pathology and Microbiology,.2000, 43(1): 17-22.

Yasmin, G., Khan, M. A., Shaheen, N., and Khan UJ. Palynological studies of Aconogonon (Polygonaceae) from Pakistan. Sarhad Journal of Agriculture. 2015, 31(1): 16-21.

Yen GC., Duh PD., Tsai CL. Relationship between antioxidant activity and maturity of peanut hulls. J Agr Food Chem. 1993, 41: 67–70.

YoshikawaK., Kokudo N., Tanaka M., Nakano T., Shibata H., Aragaki N., Higuchi T., and Hashimoto T. Novel abietane diterpenoids and aromatic compounds from *cladonia rangiferina* and their antimicrobial activity against antibiotics resistant bacteria. Chemical and Pharmaceutical Bulletin.2008, 56 (1): 89-92.

Yoshikawa M., Ninomiya K., Shimoda H., Nishida N., and Matsuda. Hepatoprotective and antioxidative properties of *Salacia reticulata*: Preventive effects of phenolic constituents on CCl_4-induced liver injury in mice. Biol Pharm Bull.2002, 25: 72–76.

Yu C., Wang CL., Jin X., Wu WK., Chan., Mckeehan ML. Increased CCl_4 induced liver injury and fibrosis in FGFR4-deficient mice. Am. J. Pathol.2010, 161: 2003-2010.

Yue SM., and Kang WY. Lowering blood lipid and hepatoprotective activity of amentoflavone from *Selaginellatamariscina in vivo*. J. Med. Plant. Res. 2011, 5: 3007-3014.

Zakaria ZA., Yahya F., Mamat SS., Mahmood D., Mohtarrudin N., Taher M., Hamid SSA., The LK., and Salle MZ. Hepatoprotective action of various partitions of methanol extract of Bauhinia purpurea leaves against paracetamolinduced liver toxicity: involvement of the antioxidant mechanisms. BMC Complementary and Alternative Medicine. 2016, 16:175. DOI 10.1186/s12906-016-1110-4.

Zhong K., LiXJ., Gou AN., Huang YN., Bu Q., and Gao H. Antioxidant and cytoprotective activities of flavonoid glycosides-rich extract from the leaves of *Zanthoxylum bungeanum*. J Food Nutr Res. 2014, 2: 349-356.