"HEPATOPROTECTIVE ACTIVITY OF *HEMIDESMUS INDICUS* IN ETHANOL INDUCED HEPATOTOXICITY"

A Dissertation Submitted to

THE TAMILNADU Dr.M.G.R MEDICAL UNIVERSITY

CHENNAI-600 032

In partial fulfilment of the requirements for the award of the Degree of

MASTER OF PHARMACY IN

BRANCH- IV- PHARMACOLOGY

Submitted By

MUHAMMED FASIL T

Register. No: 261725657

Under the guidance of

Dr. R. SURESH, M.Pharm., Ph.D.,

Professor, Department of Pharmacology

RVS College of Pharmaceutical sciences, Coimbatore-641 402

DEPARTMENT OF PHARMACOLOGY

RVS COLLEGE OF PHARMACEUTICAL SCIENCES

COIMBATORE– 641 402.

NOVEMBER 2019

CERTIFICATE

CERTIFICATE

This is to certify that this dissertation entitled **"HEPATOPROTECTIVE ACTIVITY OF *HEMIDESMUS INDICUS* IN ETHANOL INDUCED HEPATOTOXICITY"** Submitted by **MUHAMMED FASIL T (Reg. No: 261725657)** to The Tamil Nadu Dr.M.G.R Medical University, Chennai in partial fulfilment for the degree of **MASTER OF PHARMACY IN PHARMACOLOGY** is a bonafied work carried out by the candidate under guidance of **Dr. R. SURESH**, **M.Pharm., Ph.D.,** Professor, in the **DEPARTMENT OF PHARMACOLOGY**, RVS College of Pharmaceutical Sciences, Sulur, Coimbatore-641402.

Dr. D. Benito Johnson, M.Pharm., Ph.D

Station: Coimbatore

Date :

Professor and Head,
Department of Pharmacology
RVS College of Pharmaceutical Sciences,
Sulur, Coimbatore-641402.

CERTIFICATE

This is to certify that this dissertation entitled **"HEPATOPROTECTIVE ACTIVITY OF *HEMIDESMUS INDICUS* IN ETHANOL INDUCED HEPATOTOXICITY"** submitted by **MUHAMMED FASIL T (Register No: 261725657)** at the **Department of Pharmacology, R.V.S COLLEGE OF PHARMACEUTICAL SCIENCES, SULUR, COIMBATORE- 641 402** for the partial fulfilment of the university rules and regulations for the award of **MASTER OF PHARMACY IN PHARMACOLOGY,** under my guidance and supervision during the academic year 2017-2019.

Place: Coimbatore

Date:

Dr. R. SURESH, M.Pharm., Ph.D.,

Professor& Guide

Department of Pharmacology

R.V.S College of Pharmaceutical Sciences,

Sulur, Coimbatore- 641 402

CERTIFICATE

This is to certify that this dissertation entitled **"HEPATOPROTECTIVE ACTIVITY OF *HEMIDESMUS INDICUS* IN ETHANOL INDUCED HEPATOTOXICITY"** submitted by **MUHAMMED FASIL T (Reg. No: 261725657)** to The Tamil Nadu Dr. M.G.R Medical University, Chennai in partial fulfilment for the degree of **MASTER OF PHARMACY IN PHARMACOLOGY** is a bonafied work carried out under the guidance and supervision of **Dr. R. SURESH, M.Pharm., Ph.D.,** Professor, in the **DEPARTMENT OF PHARMACOLOGY**, RVS College of Pharmaceutical Sciences, Sulur, Coimbatore-641 402.

Dr. R. Venkatanarayanan, M.Pharm., Ph.D

Station: Coimbatore

Date:

Principal,
R.V.S College of Pharmaceutical Sciences,
Sulur, Coimbatore- 641 402

DECLARATION

I hereby declare that this dissertation entitled **"HEPATOPROTECTIVE ACTIVITY OF *HEMIDESMUS INDICUS* IN ETHANOL INDUCED HEPATOTOXICITY"** submitted by me, in partial fulfilment of the requirements for the degree of **MASTER OF PHARMACY IN PHARMACOLOGY** to The Tamil Nadu Dr.M.G.R Medical university, Chennai is the result of my original and independent research work carried out under the guidance of **Dr. R. SURESH, M.Pharm., Ph.D.,** Professor, in the **DEPARTMENT OF PHARMACOLOGY**, RVS College of Pharmaceutical Sciences, Sulur, Coimbatore-641402, during the academic year 2018- 2019.

Station: Coimbatore

Date:

MUHAMMED FASIL T
Reg. No: 261725657

EVALUATION CERTIFICATE

This is to certify that the dissertation work **"HEPATOPROTECTIVE ACTIVITY OF *HEMIDESMUS INDICUS* IN ETHANOL INDUCED HEPATOTOXICITY"** submitted by, **MUHAMMED FASIL T (Reg. No: 261725657)** to The Tamil Nadu Dr. M. G. R Medical University, Chennai in partial fulfilment for the Degree of **MASTER OF PHARMACY IN PHARMACOLOGY** is a bonafied work carried out during the academic year 2018-2019 by the candidate at **DEPARTMENT OF PHARMACOLOGY**, RVS College of Pharmaceutical Sciences, Coimbatore and was evaluated by us.

Examination centre:

Date:

Internal Examiner   Convener of Examination

External examiner

ACKNOWLEDGEMENT

ACKNOWLEDGEMENT

First of all I would like to thank god for his blessings to do this research work successfully. I would like to take this opportunity in expressing my deep sense of gratitude to my beloved principal **Dr. R. VENKATA NARAYANAN M.Pharm., Ph.D.,** Principal, RVS College of Pharmaceutical Sciences, under whose active guidance, innovative ideas, constant inspiration and encouragement of the work entitled **"HEPATOPROTECTIVE ACTIVITY OF *HEMIDESMUS INDICUS* IN ETHANOL INDUCED HEPATOTOXICITY"** has been carried out. I would like to take this opportunity in expressing my deep sense of gratitude to my beloved **Dr. BENITO JOHNSON, M.Pharm., Ph.D.,** Professor& Head, DEPARTMENT OF PHARMACOLOGY, RVS College of Pharmaceutical Sciences. I also take this opportunity to thank my guide **Dr. R. SURESH, M.Pharm., Ph.D.,** Professor, in the DEPARTMENT OF PHARMACOLOGY, RVS College of Pharmaceutical Sciences, Sulur, Coimbatore, for successful completion of the project.

I duly bound to all my non teaching staffs of RVS College of Pharmaceutical Sciences for their valuable advices and co-operation.

MUHAMMED FASIL T

[Reg. No: 261725657]

Table of Contents

Sl.No.	Contents	Page No.
1.	INTRODUCTION	1-20
2.	REVIEW OF LITERATURE	21-28
3.	PLANT PROFILE	29-34
4.	AIM & OBJECTIVE	35
5.	PLAN OF WORK	36
6.	MATERIALS & METHODS	37-49
7.	RESULT	50-58
8.	DISCUSSION	59
9.	SUMMARY & CONCLUSION	60
10.	BIBLIOGRAPHY	61-66

List of Figures

Figure No.	Title	Page No.
1	Structure of Human Liver	6
2	Redox Homeostasis in Liver.	15
3	The metabolic process of Ethanol in hepatocyte and the generation of ROS.	17
4	Liver Anatomy	22
5	Liver Histology	24
6	Whole *Hemidesmus indicus* plant	29
7	Plant of *Hemidesmus indicus.*	29
8	Root of *Hemidesmus indicus.*	30
9	Root of *Hemidesmus indicus.*	30
10	Flower of *Hemidesmus indicus.*	31
11	Effect of Silymarin and *Hemidesmus indicus* on serum SGPT, SGOT& ALP in Ethanol induced Liver Toxicity.	53
12	Effect of Silymarin and *Hemidesmus indicus* on serum DB& TB in Ethanol induced Liver Toxicity.	53
13	Effect of Silymarin and *Hemidesmus indicus* on total protein in Ethanol induced Liver Toxicity.	54
14	Effect of Silymarin and *Hemidesmus indicus* on LPO, GSH & CAT in Ethanol induced Liver Toxicity.	56
15	Effect of Silymarin and *Hemidesmus indicus* on Liver histology in Ethanol induced Liver Toxicity.	57-58

List of Tables

Table No.	Title	Page No.
1.	Structure of Isolated compounds from *Hemidesmus indicus.*	32
2.	Reported activities of *Hemidesmus indicus.*	33-34
3.	List of Chemicals used.	37
4.	Grouping of Animals.	42
5.	Percentage yield of Crude Extract of *Hemidesmus indicus* Root.	50
6.	Preliminary Phytochemical Screening.	50
7.	Effect of *Hemidesmus indicus* on Biochemical parameters in Ethanol induced Liver Toxicity.	52
8.	Effect of Silymarin and *Hemidesmus indicus* on LPO, SOD, GSH & CAT in Ethanol induced Liver Toxicity.	55

ABBREVATIONS

E-oH	Ethanol
ALD	Alcohol liver disease
SGPT	Alanine transaminase
SGOT	Aspartate transaminase
ALAT	Alanine aminotransferase
ASAT	Aspartate transaminase
ALP	Alkaline phosphatase
NF-Kb	Nuclear factor kappa B
Cox-2	Cyclo oxygenase 2
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase.
TNF α	Tumor necrosis factor α
CAT	Ctalase
ROS	Reactive oxygen species
SOD	Superoxide dismutase
GSH	Gluthathione reductase

Dedicated

TO

MY PARENTS

RESPECTFUL GUIDE

&

ALMIGHTY

INTRODUCTION

1. INTRODUCTION

The liver is one of the vital, largest organisms in the human body and principal site for enhanced metabolism and excretion. So it has a superior role in maintenance, performing and regulating homeostasis of the body (F Edition*et al.,* 2013). It involves almost all biochemical pathways. The major functions of the liver are carbohydrate, protein and fat metabolism, detoxification, secretion of bile and storage of vitamin. Thus, to maintain a healthy liver is a crucial factor for the overall health and well-being (Zeashan*et al.,* 2008)[1].

Liver diseases are a major global concern and this type of disorder still has extremely poor prognosis and high mortality because of the lack of effective preventive/treatment options(Wang *et al.,* 2014)[2].Some of the commonly known hepatic disorders include viral hepatitis, alcohol liver disease, non-alcoholic fatty liver disease, autoimmune liver disease, metabolic liver disease, drug-induced liver injury, gallstones, etc. which are one of the causes of morbidity and mortality in humans (Kwo*et al.,* 2016)[3]. Many types of the liver disease still have unknown causes but the most frequent liver diseases are generally caused by one of the following factors:

1.1Obesity

A fat liver is the result of the accumulation of excess fat in liver cells. Fatty tissue slowly builds up in the liver when a person's diet exceeds the amount of fat his or her body can handle. A person has a fatty liver when fat makes up at least 5-10% of the liver. The simple fatty liver can be a completely benign condition and usually does not lead to liver damage. However, once there is a buildup of simple fact, the liver becomes vulnerable to further injury, which may result in inflammation and scarring of the liver.(Petta*et al*., 2009)[4].

1.2.Alcohol

Factors such as gender, age, nationality, weight, and health can affect how a person's liver metabolizes alcohol. When the liver has too much alcohol to handle, the normal liver function may be interrupted leading to a chemical imbalance. If the liver is required to detoxify alcohol continuously, liver cells may be destroyed or altered resulting in fat deposits (fatty liver) and more seriously, either inflammation (alcoholic hepatitis) and/or permanent scarring (cirrhosis).(Mota*et al.,* 2010).

1.3. Drugs and toxins

More than 900 drugs, toxins, and herbs have been reported to cause hepatotoxicity. Chemicals and drugs used to induce hepatotoxicity include Ethanol, CCl4, Galactosamine, Cadmium, Paracetamol, Isoniazid, Rifampicin etc.The liver is responsible for processing most of the chemicals and medications that enter the body – this leaves it vulnerable to acute or chronic liver disease caused by chemicals. In some cases, this is a predictable consequence of overexposure or over-consumption of certain chemicals such as acetaminophen or industrial toxins like polyvinyl chloride or carbon tetrachloride. In other cases, chemicals can cause an unpredictable reaction.(Tsui*et al.,* 2003).

1.4.Genetics

Several forms of liver disease are caused by defective genes. These forms of the liver disease may be diagnosed in infancy or may not show up until later in life. Examples include hemochromatosis, Wilson disease, tyrosinemia, alpha 1 antitrypsin deficiency, and Glycogen Storage disease((Scorza*et al.*,2014).

1.5. Autoimmune disorders

Sometimes a body's immune system may begin to attack the liver or bile ducts causing inflammation and scarring which leads to a progressive form of liver disease. Examples of liver diseases believed to be caused by the immune system are primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC) and autoimmune hepatitis.(Mirzaagha*et al.,* 2010)[5].

1.6.Cancer

Although primary liver cancer is relatively uncommon, many other forms of cancer often metastasize in the liver. Because the liver filters a high volume of blood which may be carrying cancer cells, it is susceptible to developing a form of secondary cancer. If cancer originates in the liver, it is often caused by hepatitis B, hepatitis C or it can develop in cases of advanced liver disease when cirrhosis is present(Ariff*et al.,* 2009)[6].

1.7. Viral hepatitis

Caused by viruses that attack the liver, viral hepatitis comes in many forms. The most common forms world-wide are hepatitis A, B and C. Although hepatitis A and B can be prevented by a vaccine, there is no vaccine for hepatitis C. According to

WHO estimates, globally 170 million people are chronically infected with hepatitis-C alone and every year 3–4 million are newly added to the list. Also, there are more than 2 billion infected by hepatitis B virus (HBV) and over 5 million are getting infected with acute HBV annually (Negi*et al.,* 2007)[7].

The association between alcohol abuse and liver disease has been recognized throughout the history of medicine. During the first half of the century, the concept prevailed that the liver disease was not due to the toxicity of alcohol but rather to the dietary deficiencies commonly associated with alcoholism. Such a view was most clearly formulated by Best et al, 2016. Since then three decades of epidemiologic, clinical and experimental studies have clearly established the role of the hepatotoxicity of alcohol, in addition to the influence of dietary factors. The highest amount of alcohol per adult was reported in Europe, especially in Russia and surrounding countries, and the least was in the mostly Islamic regions of the Eastern Mediterranean and in the less developed region of Southeast Asia, predominantly India (Obot et al., 2005)[8]. There is no effective therapy for the alcohol-induced liver disease.

Recent reports on hepatotoxicity suggest that both oxidative stress and abnormal cytokine production, especially tumor necrosis factor (TNF), play important etiological roles in the pathogenesis of ALD. Therefore, agents that has both antioxidant and anti-inflammatory properties, particularly anti-TNF production, represent promising therapeutic interventions for ALD (Li *et al.,* 2016).

Hepatotoxicity is the leading cause of withdrawals of many drugs from the market. Synthetic drugs for the treatment of liver injury can further cause liver damage, so herbal medication is the only way to cure the hepatic injury. Hence there is a growing trend in the investigation of phytoconstituents for hepatoprotective.Various plant extracts and polyherbal medications have been clinically approved for their potent hepatoprotective activity. Many active plant extracts are frequently utilized to treat a wide variety of clinical diseases including liver disease. Therefore, searching for effective and safe drugs for liver disorders are continues to be an area of interest. (Ublick et al.,2017)[9].

The use of natural remedies for the treatment of liver diseases has a long history, starting with the Ayurvedic treatment, and extending to the Chinese, European and other systems of traditional medicines. A large number of plants and

formulations have been claimed to have hepatoprotective activity. Nearly 160 phytoconstituents from 101 plants have been claimed to possess liver protecting activity. Liver-protective herbal drugs contain a variety of chemical constituents like phenols, coumarins, lignans, essential oil, monoterpenes, carotenoids, glycosides, flavanoids, organic acids, lipids, alkaloids, and xanthones derivatives(Pandey*et al.,* 2014).

The previous investigations have revealed that hepatoprotective effects are linked with natural antioxidants. Commonly used plants in the herbal formulation in India includes *Andrographis paniculata*, *Boerhaavia diffusa*, *Eclipataalba, Picrorrhizakurroa, Olednlandiacorymbosa, Asteracanthalongifolia*, Apiumgraveolens, *Cassia occidentalis*, *Cichoriumintybus*, *Embeliaribes*, *Curcuma longa* and *Tageteserecta*(Madrigal *et al.,*2014)[10].

Hepatoprotective activity of the ethanol extract of *Curcuma longa* was studied against paracetamol-induced liver damage in rats (Salama*et al.,* 2013)[11]. Hepatoprotective of Panduratin against liver damage was studied (Salama*et al.,* 2018)[12]. Hepatoprotective and immunomodulatory properties of aqueous extract of Curcuma longa in carbon tetra chloride intoxicated Swiss albino mice were studied (Sengupta et al., 2011)[13]. Protective effects of quercetin on liver injury induced by ethanol were studied (Chen et al. 2010)[14].

Bose et al., 2009, reported the hepatoprotective activity in flowers of *Tageteserecta* by carbon tetrachloride-induced hepatopathy model. The ethanolic extract showed the increase in serum AST, AST, ALP,and bilirubin levels. Phytoconstituents such asflavonoids, terpenoids,and steroids are shown to be responsible for the observed hepatoprotective activity.

Reactive oxygen species and free radicals plays important role in the etiology of various diseases like inflammation, cataract, rheumatism, atherosclerosis, arthritis, ischemia, reperfusion injury including liver disorders.

Hemidesmus indicus(Asclepiadaceae) known as Indian Sarsaparilla, or Anantmul, is a well known and very common plant in Ayurvedic system of medicine. The plant is found throughout in India in plain and low hills area[15]. The root is sweet bitter, cooling, and aphrodisiac, antipyretic and also it cures leprosy, leucodermia, bronchitis, asthma and general debility[16, 17]. Traditionally it is used as diuretic, antirheumatic, blood purifier and antidote for snake bite[18]. It has been used as a

traditional medicine for the treatment of biliousness, blood diseases, diarrhea, respiratory disorders, skin diseases, syphilis, fever, bronchitis, asthma, eye diseases, epileptic fits in children, kidney and urinary disorders, loss of appetite, burning sensation and rheumatism. Jain and Singh reported that Hemidesmus indicus employed in traditional medicine for gastric ailments. *Hemidesmus indicus* extract mainly consist of essential oils and phytosterols, such as hemidesmol, hemidesterol and saponins. The active principle of *Hemidesmus indicus* is 2-hydroxy 4-methoxy benzoic acid (HMBA).

Roots are reported to have anti-inflammatory[19, 20] and antimicrobial[21] properties. Methanolic extract of root bark of *Hemidesmus indicus* shows protection against Isoniazide and Rifampicin induced hepatic damages[22]. Root bark also reported to possess antioxidant activity.

1.8. LIVER

The liver is the largest solid organ in the body. In adults, the livers can weigh up to 1.5 kg. It is in the upper-right abdomen, just under the rib cage and below the diaphragm (the thin muscle below the lungs and heart that separates the chest cavity from the abdomen). The liver is part of the digestive system. Liver acts as a giant chemical processing plant in the body and are central in the regulation of body homeostasis (Irawanet al., 2015)[23].

1.9. STRUCTURE

The human liver primarily consists of two lobes the larger right lobe and the smaller left lobe and many lobules contain hepatocytes, blood sinusoids and bile ducts. The lobes are separated by a band of tissue called the falciform ligament (also) called the broad ligament), which helps attach the liver to the diaphragm. A layer ofonnective tissue, called Glisson’s capsule or the capsule, covers the liver. Each lobule is passed through by central hepatic vein and surrounded with hepatic artery and portal vein, which faciliates a tight communication between liver and both vascular and lymphatic system (Rajasekaran.*et al.,* 2009)[24], (Sanjay KJ*et al.,* 2013)[25].

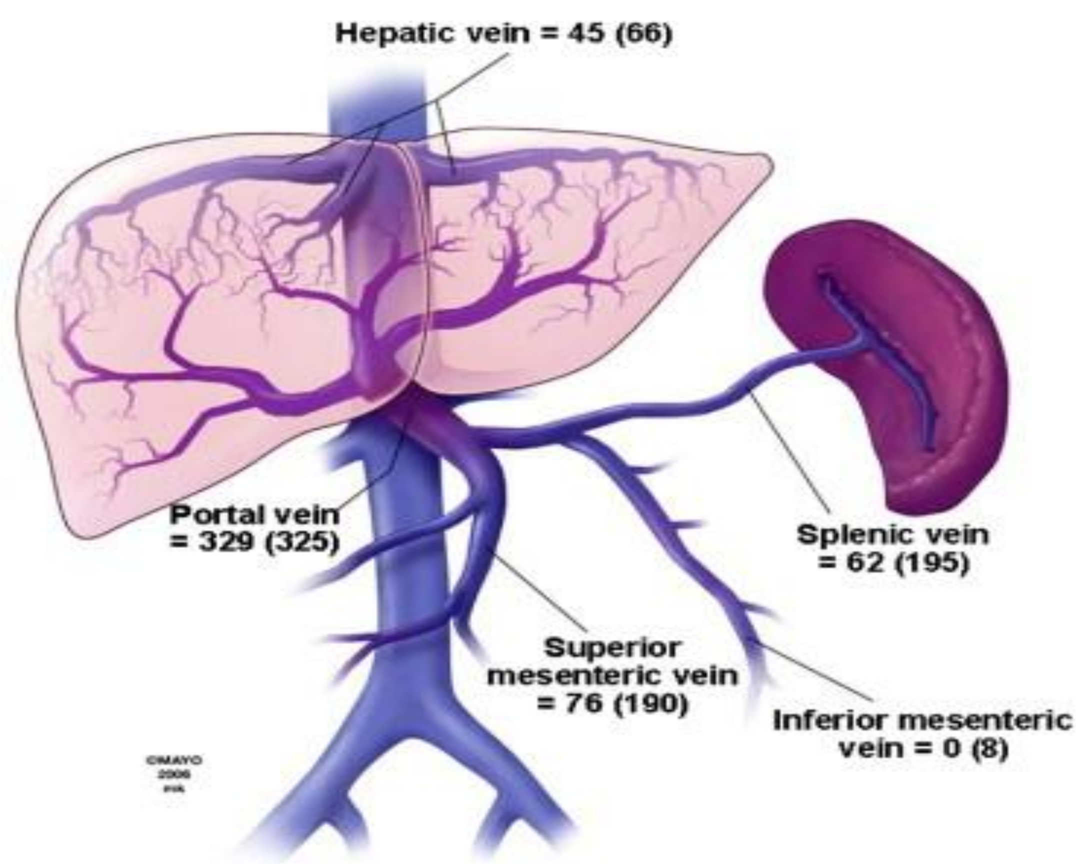


Figure No. 1 Structure of human Liver

1.10. Cells of liver

The hepatocytes are liver parenchymal cells which consist of 60-80% of liver cell population. Whereas, the cells such as kupffer cells, stellate cells, biliary epithelial cells, sinusoidal endothelial cells and lymphocytes are non-parenchymal cells and comprise the rest 20-40% of the liver cell population (Racanelli*et al.*, 2006)[26].

Hepatocytes compose a major fraction of hepatic cell population. They are polyhedral in shape, arranged in single-cell cords or plates. These hepatocytes are linked together via intercellular adhesion complexes and tight junctions. Their one side faces the persinusoidal space, while the other faces the bile canaliculi and covered with microvilli (Bioulac-Sage et al. 2007)[27]. Binucleated cells with large polyploidy nuclei are commonly seen in adult liver (Abel T*et al.,* 2013). Hepatocytes are responsible for most of the liver functions such as metabolism, detoxification, synthesis, and storage of nutrients, carbohydrates, fats, and vitamins. They are also involved in secretory and excretory functions along with other hepatic cells. These

functions are performed by different hepatocytes residing in different zones of hepatic lobules (Duncan *et al.,* 2009)[28].

Kupffer cells are resident macrophages in liver with largest population. They are attached to the luminal surface of the sinusoidal endothelium **.**These cells are essential for the phagocytosis of foreign particles, infecting organism as well as cytokines products (Taub*et al.,* 2004)[29].

Hepatic stellate cells are located within persinusoidal space of Disse, in the recesses between hepatocytes. These cells are associated with several functions such as secretion of cytokines, storage of vitamin A and synthesis of hepatic extracellular matrix. They get activated during liver injury and play a key role in progression of fibrosis (Wang Y*et al.,* 2013)[30].

Biliary epithelial cells, line the bile duct in portal triads and are also known as the cholangiocytes. The connecting duct between bile duct and bile canaliculi (canal of Hering) along with hepatocytes are also lined by these biliary cells. They are involved in modifying bile composition by altering solute and water content (Katsuda *et al.,* 2013).

Endothelial cells are largest group of non-parenchymal cells of liver and line the intrahepatic circulatory vessels of liver and provide a large surface area for nutrient absorption. They form a pathogenic and selective barrier during separation of hepatocytes from sinusoidal blood by exchange of molecules (Racanelli and Rehermann*et al.,* 2006, Taub*et al.,* 2004).

Lymphocytes are present everywhere in the liver parenchymal sinusoids. These lymphocytes are also a part of innate immune system and selectively rich in NKTcells and natural killer cells providing defence against invading pathogens (Taub et al., 2004).

1.11. Liver as a secretory organ

Bile salts are required for emulsification of dietary fats prior to their absorption and digestion. These bile salts are present in bile juice that is produced by the liver and secreted into duodenum by common bile duct. Many endogenous and exogenous compounds are known to be metabolized and excreted into bile through liver. Limited amount of these compounds were reabsorbed in the small intestine and eliminated bythe kidney. For example, recycling of iron while globins chains get catabolized and their components are reused. However, hemoglobin also contains a

porphyrin called heme that must be eliminated. Heme is converted into free bilirubin inside phagocytic cells, which is released into plasma and secreted by the liver. It also involved in bile excretion (Jones*et al.,* 1977).

1.12. Liver as an immuno-organ

Liver retains the important immunological functions with some new ones (Crispe*et al.,* 2009). These functions belong to both innate and adaptive immunity branches. For example, production of acute phase proteins, non-specific phagocytosis & cell killing, disposal of inflammatory wastes and non-specific immunoregulation are some of the innate immune functions. On the other hand, the adaptive immune responses of T-cells are reviewed extensively such as deletion of activated T-cells, induction of tolerance to ingested and self-antigens, extrathymic proliferation of T cells and specific immunoregulation (Crispe*et al.,* 2009)[31].

1.13. Liver as a metabolic organ

Hepatocytes are associated with metabolic process in the body. They also play a critical role in synthesizing molecules that support homeostasis and regulating energy balances (Felber and Golay*et al.,* 1995).

- **Carbohydrate metabolism**

Critical concentrations of glucose must be maintained inside body. This is an important function of the liver. Hepatocytes are residence for different metabolic pathways and enzymes that regulate blood glucose level (Chanda *et al.,* 1995). Excess glucose enters into the blood after intake of food and rapid synthesis of glycogen via glycogenesis occurs in liver. Whereas when glucose concentration decreases in blood, the liver activates another pathway which leads to depolymerization of glycogen (glycogenolysis) into glucose. When hepatic glycogens reserves become exhauste, during prolong starvation, hepatocyte activates gluconeogenesis in order to complete glucose demand of body (Chanda*et al.,* 1996)[32].

- **Fat metabolism**

Lipid metabolism is predominantly carried out by the liver. Triglycerides oxidized to produce energy inside liver. The hepatocytes are site for breakdown of many fatty acids into acetoacetate. Liver also associated with synthesis of lipoproteins,cholesterol, and phospholipids. Excess carbohydrates and proteins get converted into fatty acids and triglyceride in liver, and later on exported and stored in adipose tissue (Kulkarni SR *et al.,* 2013).

- **Protein metabolism**

The hepatocytes are responsible for synthesis of most of the plasma proteins such as albumin and clotting factors necessary for blood coagulation. Liver is also involved indeamination and transamination of amino acids. Liver is also associated withconversion of the non-nitrogenous part of those molecules to glucose or lipids. Liver is responsible for synthesis of urea from ammonia which is very toxic and may result in diseases associated with central nervous system (Rebelato*et al.,* 2013).

1.14. Liver: pathological condition

Beside its main function such as storage and distribution of nutrients, liver also function as detoxifying organ. The liver takes up chemical substances ingested through the food and absorbed through the gastrointestinal tract. Doing so, however, harbours the danger that the substances which are degraded and/or eliminated by liver lead to tissue damage (Ramadori *et al.,* 2008). Most of these substances are compatible with cellular metabolic processing thus utilized by body intact or can be transformed further into components of cells and tissues. But substances which are not processed further or metabolized completely enter into portal circulation and processed by hepatocyte so that they can be excreted out of body.

These un-metabolized substances known as xenobiotics are processed by enzymes such as members of the family of cytochromeP450 enzymes. This detoxification process generates free radicals and reactive intermediate reacts with DNA and proteins, forming adducts and causing hepatocyte damage and/or cell death. Liver get exposed to several compounds such as acetaminophenon, carbontetrachloride, thioacetamide and various other chemical carcinogens in the process of detoxification. Depending upon the quantity of toxic compounds, an acute, or a chronic injury occurs to the liver. They commonly damage hepatocytes (main functional cell of liver) and under chronic condition death occurs (Fausto, 2000*et al.,* 2011).

1.15. The liver and affecting diseases

Being the main detoxifying and metabolizing organ, liver is prone to a number of diseases. Severity of diseases depends on the onset and duration of pathologicalconditions. On the bases of it diseases can be categorised into acute and chronic. They are characterised into, hepatic steatosis, jaundice, hepatitis, fibrosis,

cirrhosis,cholestasis and cancer (hepatocellular carcinoma and cholangiocarcinoma). Some common pathological conditions are described below.

- **Hepatic steatosis**

Is conditions were triglycerides accumulated in hepatic cells increase abnormally leading to fatty liver disease. The hepatocytes maintain triglyceride content of body through normal processes of uptake, synthesis, and esterification of fatty acids. Beside many metabolic, nutritional and genetic cause of high triglyceride level in body it majorly due to excessive alcohol intake (AFLD) or obesity-related causes (NAFLD).Severe steatosis or fatty liver associated with inflammation known as steatohepatitis(Day and James, 1998; den Boer *et al.,* 2004; Reddy and Rao, 2006).

- **Jaundice**

Is characterized by overload of bile pigment on liver cells and condition is Also known as hyperbilirubinemia. It may also result from disturbances in uptake, transport, conjugation, and biliary excretion mechanisms of bilirubin by the liver cells. It can be caused due to damage, injury, or infection to the liver cells. However, failure of biliary excretion and cholestasis (intra-hepatic or extra-hepatic) may lead to jaundice (Sherlock*et al.,* 1967).

- **Hepatitis**

Is characterized by inflammation of the liver. It can be caused by various viruses, drugs/toxins, alcohol, and autoimmunity conditions. Hepatitis viruses such as A, B, C, D and E, are commonly known viruses. These viruses differ in their modes of transmission. Hepatitis A and E viruses are transmitted by contaminated food and water. Whereas hepatitis viruses B, C and D are get spread through potential contact with infected body fluids. Hepatitis can be acute or chronic depending upon its severity. Viruses of type B and C can cause chronic hepatitis while all other are associated with acute hepatitis. Chronic hepatitis leads to liver cirrhosis, whichultimately leads to hepatocellular carcinoma. Hepatitis may lead to cholestasis in some cases (Ferrell*et al.,* 2000, Popper*et al.,* 1972; Waris and Siddiqui, 2003).Fibrosis is characterized by encapsulation or replacement of injured liver tissue by a collagenous scar. It occurs during normal wound-healing response, resulting in an abnormal continuation of fibrogenesis. This includes deposition and production of connective tissue/matrix protein (Bataller eta l., 2005).

- **Cirrhosis**

Is an advanced stage of liver fibrosis. It involves distortion of the hepatic vasculature. Cirrhosis can be defined as widespread hepatic fibrosis with nodule formation. It leads to the distortion of hepatic vessel; impair blood supply, with scar in the space of Disse and endothelial fenestrations are lost. The impaired hepatocyte function, exhaustive proliferation and division, are common feature of cirrhosis. The chronic liver diseases conditions that result in cirrhosis include alcohol abuse, hepatitis B and C, autoimmune hepatitis. It also caused due to continued exposure to certain toxins/drugs that lead to hepatocyte necrosis/apoptosis (Schuppan and Afdhal, 2008; Sherlock*et al.,* 1968).

1.16. Patterns of gene expression and liver regeneration

Drug induced hepatotoxicity causes sensible amount of mortality and morbidity. But due to insufficient information regarding mechanism of drug induced liver injury it becomes a major concern in drug development. Over the last decade, several gene expression studies using microarray technology have focussed on three generation phenomenon that follows drug induced liver injury in rodents (Togo*et al.,* 2004; Xu *et al.,* 2005). The most important changes in expression profile of genes occur during the priming phase of regeneration. It helps in the categorization of expression patterns into immediate-early and delayed-early genes (Haber *et al.,* 1993). More than 100 genes which are not expressed in normal liver get induced during regenerative process; responsible for entry of hepatocytes into cell cycle and play an essential role in fulfilling metabolic demand of cells while dividing (Michalopoulos*et al.,* 2007).

Genes associated with priming phase of regeneration include genes for transcription factors, stress and inflammatory responses. These genes regulate entry of liver cells into cell-cycle. Genes involved in cytoskeletal and ECM remodelling is also activated during priming phase (Su etal. 2002). The G1/S transition, involves a set of differentially expressed genes with many diverse functions (Satyanarayana *et al.,* 2004). Data analyses of expression pattern of genes with respect to different phases of regeneration, shown up-regulation of genes involved in protein synthesis, cytoskeletal organization and DNA replication while down-regulation of steroid and lipid metabolism genes(Fukuhara *et al.,* 2003).Although microarray technology provides a global view of gene expression pattern that are either and/or both

hepatocytes and non-parenchymal cells. Few efforts have been made to study gene expression of every liver cell (Haber *et al.,* 1993).Most recent studies have monitored gene expression changes in both Kupffer cells(Xu CS*et al.,* 2012) and sinusoidal endothelial cells especially during liver regeneration(Xu CS *et al.,* 2011).

1.17. Pharmacological animal models

Mammalian liver is prone to a large number of toxins and drugs, and get influenced by them in various manners. In contrast to surgical animal models, chemically induced liver injury models are easier to work upon and provide a better way to study many related liver diseases in humans. In these models, regenerative response often varies with dose and mode of administration of drug. A particular amount of drug dose induced acute liver failure while repeated doses can induce chronic injury suchas liver cirrhosis (Palmes and Spiegel, 2004).It must be noted that different drugs can have different effects on the liver tissue depending on their metabolic process and the mechanisms of regeneration which differ to a large extent. In these kind of toxic models the processes of liver injury and repair are overlapped and/or closely associated. Effects of some commonly employed chemicals related to liver damage and injury are discussed below.

These chemicals have similar mode of action to induce centrilobular hepatic damage. The hepatocytes nearer the portal triads receive oxygenated blood and nutrients thus are less prone to injury, while more distal hepatocytes receiving less oxygen and nutrients, and are more prone to ischaemic or nutritional damage (Rahman *et al.,* 2002).The mode of action of these chemicals depends upon the bioactivation by liver cytochrome P-450system. Thus, these hepatocytes surrounding the central vein become the prime targets for the damage induced by such chemicals (Sturgill*et al.,* 1997).

- **Carbon tetrachloride (CCl4)**

A classical hepato toxicant is used to induce liver injury by breakdown of its metabolic byproduct through cytochrome P-450 enzyme. Trichloromethyl is a highly reactive metabolite of CCl4 and damages hepatocyte by triggering lipid peroxidation. Kupffer cells get activated by free redicals and help in progression of injury by release of cytokines. Acute, reversible liver injury can be induced by a single oral, intraperitoneal, or subcutaneous dose of CCl4 during fibrosis Rao *et al.,* 1997; Taniguchi *et al.,* 2004; Weber *et al.,* 2003).

- **Thioacetamide (TA)**

A classical hepatotoxicant is commonly used to induce acute/chronic liver injury depending on dose and time. Thioacetamide generally induces centrilobular necrosis. Thioacetamide undergoes bioactivation by cytochrome P2E1into TA sulfoxide and finally to TA-S, S-dioxide which damage to liver tissue (Mangipudy and Mehendale, 1998) detailed in section below.

- **D-galactosamine**

Induces hepatotoxicity by causing intracellular deficiency of uridine metabolites. Other factors such as endotoxinaemia are also associated with it. It is responsible for inducing fulminant liver failure and liver damage. Liver regeneration induced by D-galactosamine is weaker as compared to CCl4 induced liver regeneration (Kalpana *et al.,* 1999; Keppler *et al.,* 1968;)[33] Reutter *et al.,* 1968).Acetaminophen is most commonly used drug to induce liver intoxication causing acute liver failure. Normally, acetaminophen undergoes biotransformation in the liver by a combination of glucuronidation and sulphation. They are excreted from body via kidney. An overdose of acetaminophen may block its breakdown pathway thus cytochrome P-450 oxidase system helps in acetaminophen metabolism (L M*et al.,* 2013).

- **Ethanol**

Is a hepatotoxicant which damages hepatocyte. It is mainly associated with liver steatosis and necrosis. Different modes of administration cause variation in results (Palmes*et al.,* 2004). Long-term exposure may leads to activation of progenitor cells and involvement of LPS-driven pathway (Roskams *et al.,* 2003; Smith *et al.,* 1996).

- **Hepatotoxins**

Hepatotoxicity refers to liver dysfunction or liver damage that is associated with an overload of drugs or xenobiotics. Chemicals that cause liver injury are called hepatotoxins. There are literally thousands of chemicals that could be toxic to the liver and a few examples of these chemicals include: Carbon tetrachloride,Thioacetamide, Galactosamine, Alcohol, Paracetamol, Antitubercular drugs(Rifampicin, Isoniazid and pyrazinamide) ,Azathioprine, Ranitidine etc(Farias, M.S et al.,2012)[34].

1.17. ETHANOL

Alcohol is widely consumed in alcoholic drinks in modern society, and ethanol is one of the main causes of a variety of medical problems and liver diseases worldwide [Abdul-Hussain et al., 1991][35]. The liver is the major target organ of ethanol toxicity [Arvind S *et al.,* 2007].Chronic ethanol feeding causes a decrease in the major antioxidant factors in the liver, including enzymes [Beers RF eta.,1952, Bhandarkar,M.R *et al.,* 2004][36, 37] and non enzymatic antioxidants [Bhandarkar, M.R et al.,2006][38]. This is due to the generation of an excessive amount of reactive oxygen species (ROS), which results in the detrimental effects of the cellular antioxidant defense system [Cartwright *et al.,* 2008][39]. Thus, excess alcohol consumption may accelerate an oxidative mechanism directly or indirectly, which eventually produces cell death and tissue damage (Chattopadhyay R.R et al., 2003[40] – Farghali H et al 1999). Oxidative stress and the subsequent formation of reactive oxygen species have been correlated with a number of diseases in animal models and humans. It is well documented that chronic ethanol consumption induces oxidative stress in the liver (Esterbaur et al., 1991). Alcohol liver disease (ALD) is the most common form of liver dysfunction in the world. ALD is also the major cause of chronic diseases and death associated with alcohol misuse (French, 1996). The liver accounts for 90% of alcohol metabolism and is the organ most adversely affected (Kaviarasan *et al.,* 2007). Ethanol metabolism gives rise to the generation of excess amounts of reactive oxygen species which causes a profound increase in hepatic lipid synthesis and has a detrimental effect on the cellular antioxidant defence system (Navasumrit *et al.,* 2000; Ozaras *et al.,* 2003).

The principal damage of ethanol are hepatic induced by lipid peroxidation, decreasing activities of antioxidant enzymes (such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) and generation of free radicals, as well as elevation of hepatic enzymes such as alanine aminotransferase (ALT), aspartate aminotransferase (AST) and alkaline phosphatase (ALP) (Escobar *et al.,* 1996; O¨ ner *et al.,* 2008). ALT and AST are the most sensitive biomarkers employed in the diagnosis of hepatic damage. Liver damage is a widespread pathology, which in most cases involves oxidative stress and is characterized by a progressive evolution from steatosis to chronic hepatitis, fibrosis and cirrhosis. Various xenobiotics including ethanol are known to cause hepatotoxicity (Grant *et al.,* 1988; Lieber, 1997,

2005). A number of studies suggested that ethanol toxicity is associated with elevated generation of reactive oxygen species (ROS) (Das and Vasudevan, 2007) and concomitant reduction in endogenous antioxidant capacity (Bergamini *et al.,* 2004; Lieber, 2005). Ethanol alters the antioxidant profile of the liver, including the antioxidant enzymes like superoxidedismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione transferase (GST) (Grissa *et al.,* 2007). Steroids, vaccines and antiviral drugs, used as therapies for liver pathologies, have potential adverse side-effects, especially if administered chronically or subchronically. Therefore, herbal products and traditional medicines with better effectiveness andsafe profiles are needed as a substitute for chemical therapeutics. As oxidative stress plays a central role in liver pathologies and their progression, the use of antioxidants have been proposed as therapeutic agents as well as drug coadjuvants to counteract liver damage. Recent studies have shown that various herbal extracts, having hepatoprotective effect (Mohamed Saleem *et al.,* 2010), could protect liver against oxidative stress by altering the levels of increased lipid peroxidation (LPO) and by enhancing the decreased activities of antioxidant enzymes such as SOD, CAT and GPx (Hua *et al.,* 2010; Jain *et al.,* 2008; Soussi *et al.,* 2009).

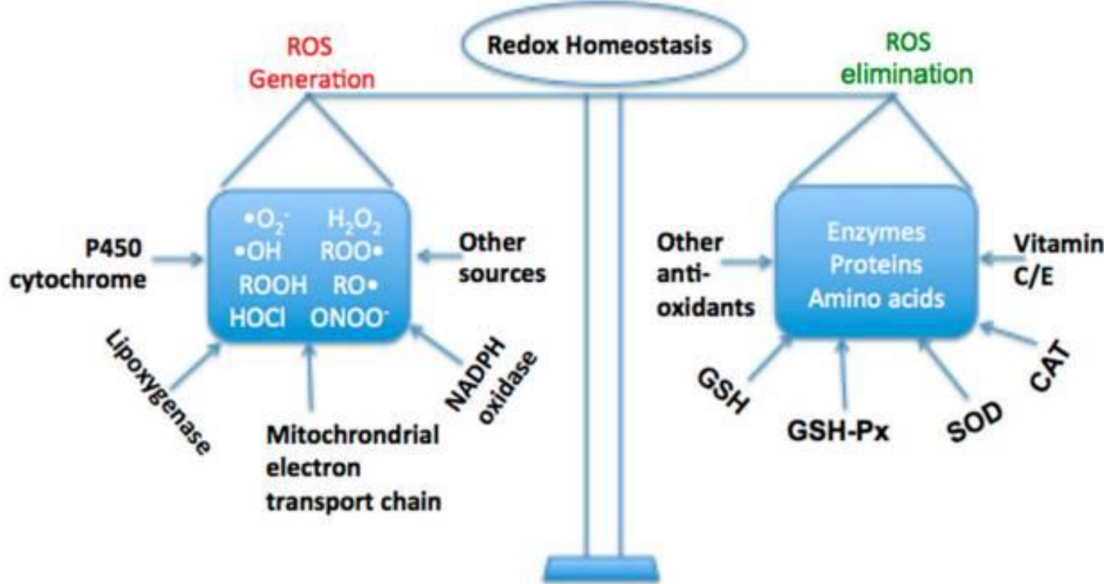


Figure No: 2 Redox homeostasis in liver

1.18. Oxidative Stress Caused by Alcohol

Alcohol beverages are widely consumed all over the world; however, excessive alcohol consumption may cause a series of health problems. It was reported

that alcohol consumption accounting for an estimated 3.8% of global mortality. Alcoholic liver disease (ALD) is one of the most important causes of liver-related death, which is associated with increased dos and time of alcohol intake. In 2003, it has been reported that age- and sex-adjusted mortality rate of ALD was 4.4/100,000. Although reductions in overall ALD mortality were observed in several reports on a country scale, it is more likely due to advances in disease management rather than a decrease in the prevalence of ALD, which could be supported by increases in hospital admissions for alcoholic hepatic failure and alcoholic hepatitis (Louvet, A. *et al.,* 2015– Zhu, H et al., 2012). ALD may progress from steatosis to more severe liver diseases form, such as hepatitis, fibrosis, and cirrhosis (Banerjee, P.*et al.,* 2013, Gao, B et al., 2016). As a matter of fact, more than 90% heavy drinkers develops fatty liver, and about 30% of heavy drinkers further develops advance forms of ALD. Although pathogenesis of ALD has not been fully elaborated, the direct consequence of ethanol metabolism seems to be related to ROS production, mitochondrial injury and steatosis, which are the common features of acute and chronic alcohol exposure (C.J. Mechanisms *et al.,* 2010, Diehl, A.M *et al.,* 2005).

It is well illustrated that at least three distinct enzymatic pathways are involved in the process of ethanol oxidation (Wang, H *et al.,* 2015). The primary pathway for the ethanol metabolism is dehydrogenase system. It is initiated by alcohol dehydrogenase (ADH), a NAD+-requiring enzyme expressed at high levels in hepatocytes, which oxidizes ethanol to acetaldehyde. Then, acetaldehyde enters the mitochondria where it is oxidized to acetate by aldehyde dehydrogenases (ALDH). The second major pathway to oxidize ethanol is the microsomal ethanol oxidizing system (MEOS), which involves an NADPH-requiring enzyme, the cytochrome P450 enzyme CYP2E1. The MEOS pathway is prompted in individuals who consume alcohol chronically. In addition, infrequently, ethanol can also be oxidized by catalase in peroxisomes. Since this oxidation pathway requires the presence hydrogen peroxide (H2O2), under normal conditions, this pathway plays no major role in alcohol metabolism [Wang, H *et al.,* 2015–Lakshmanan, J. *et al.,* 2013]. During the metabolism processes via dehydrogenase system and MEOS system, NADH or NADP+ will be produced in bulk, leading to the increase of ROS, which cause oxidative stress resulting in hepatocyte injury, and finally trigger various livers.

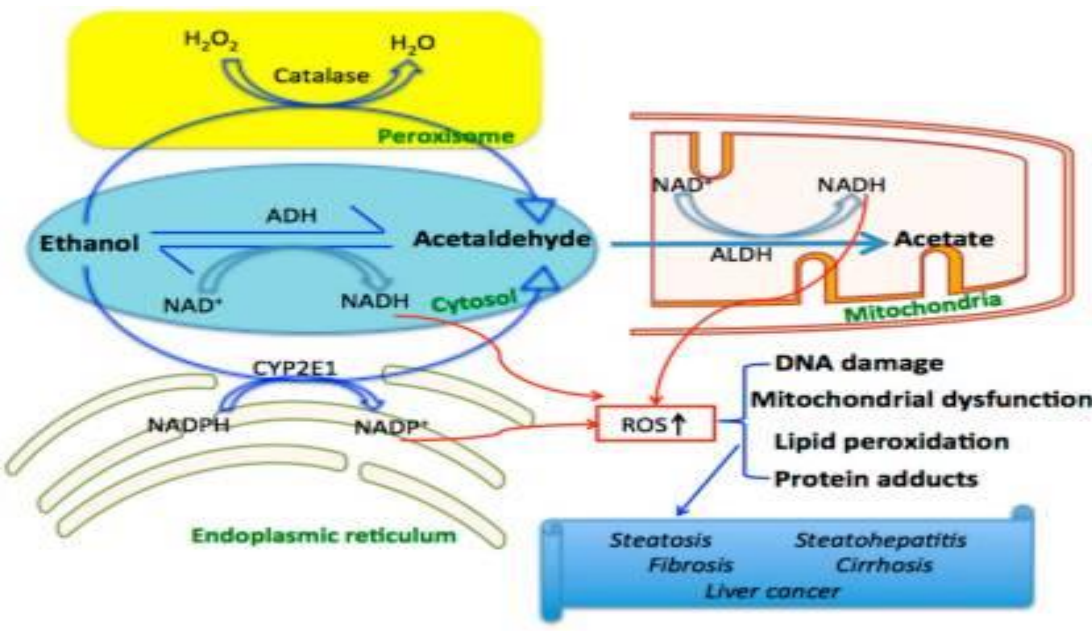


Figure No.3 The metabolic process of ethanol in hepatocyte and the generation of ROS.

Studies have demonstrated that enzymatic as well as non-enzymatic systems which maintaining cellular homeostasis is remarkably affected by alcohol in diverse models. In particular, the activities of SOD, CAT, GSH-Px, GRD, and GST, as well as the level of lipid peroxidation were changed in animals treated with alcohol (Shanmugam, K.R.; *et al.,* 2016–Dahiru, D.; *et al.,* 2007).

For example, SOD and CAT activities were decreased and the lipid peroxidation level was significantly increased in the liver of 30 days alcohol-treated diabetic rats (Shanmugam, *et al.,* 2016). An increase of lipid peroxidation and hepatic cytochrome P450, and decrease of hepatic SOD, GSH-Px, GRD, GST, and GSH were also observed in mice treated with dimethoate in combination with ethanol (Babczynska, et al., 2006). Furthermore, oxidative stress and antioxidant enzyme were measured in patients with ALD (Masalkar, *et al.,* 2005). It was found that as the severity of the disease increased, followed by elevation of serum level of lipid peroxidation indicator malondialdehyde (MDA) and the concentrations of serum vitamins E and C, which act as indexes of antioxidant status, were decreased in ALD patients. The pro-oxidant and antioxidant status in chronic alcoholics have been detected in several studies.

The significant decreases of GSH levels in liver and blood of patients with alcoholic liver disease were observed when compared to controls. However, the activity/content of SOD and CAT after alcohol exposure is rather controversial, with

reports of increases, no changes, or decreases, depending on the amount and time of alcohol consumption (Balakrishnan, *et al.,* 2008, Videla, L.A.et al., 2004). Nevertheless, the increased oxidative stress in patients with ALD has been demonstrated. It was argued that the increases of antioxidants enzymes such as SOD, CAT and GSH-Px might be a compensatory regulatory response to increased oxidative stress (Chen, Y.L et al., 2011). The level of ALT was increased significantly while the level of AST was decreased significantly in patients with ALD (Albano, E *et al.,* 2008, Videla, L.A *et al.,* 2009).

1.19. PLANTS SHOWING HEPATOPROTECTIVE ACTIVITY

- ***Zanthoxy lumarmatum***

This plant is widely used as hepatic tonic for many liver problems. It is reported that of ethanolic extracts of bark have hepatoprotective activity against CCl4 induced hepatic damage (Tan, P.V. *et al.,* 2003).

- ***Andrographis paniculata (Acanthaceae)***

Andrographolide is an active constituent extracted and isolated from *Andrographis paniculata*. Ex vivo, the compound illustrates a considerable dose dependent protective activity against paracetamol-induced toxicity on isolated rat hepatocytes upon administration of andrograoholide(Djehuty, M. et al.,2007)[41]. The results clearly depicted *Andrographis paniculata* extract to exert a choleretic effect that reduces the cholestasis and diminishes retention as well as increase the excretion of toxic xenobiotics from liver. Further, it also stimulated immune system to fight against inflammation, is mediated from the release of cytokinin from immunomodulators(Cristobal, M. *et al.,* 2000).

- ***Azadirachta indica (Meliaceae)***

The hepatoprotective activity of *Azadirachta indica* against paracetamol induce hepatic damage in rats by studies on antioxidant enzymes, (Glutathione peroxidase(GPX), glutathione-S-transferase (GST), superoxide dismutase (SOD) and catalase(CAT). The results suggest that the hepatoprotective effect exerted by neem may possibly be due to its ability to exert antioxidative effect via augmenting the level of hepatic antioxidant enzymes (Cristobal, M. *et al.,* 2000).

- ***Allium sativum (Liliaceae)***

The hepatoprotective effects of *Allium sativum* (Garlic) were studied by on experimental rats exposed to lead for one week. Results depicted that

administration of lead in rats caused some level of liver or hepatic damage in the animals and that post-lead treatment with A. sativum exerted some hepatoprotective effects (Crozier, A. et al., 2000).

- ***Boerhavia diffusa (Nyctaginaceae)***

The roots of *Boerhavia diffusa*, are used by a large number of tribes in India for the treatment of various hepatic disorders and for internal inflammation (Jovanovc, M. *et al.,* 2005). Punarnava contains alkaloids named as punarnavine and punarnavoside which shows antifibrinolytic activity but the hepatoprotective activity has been attributed to ursolic acid. Keppler and co-workers demonstrated that Ursolic acid isolated from the leaves showed a dose dependent (5-20 mg/kg) hepatoprotective activity (21-l00%) in rats against thioacetamide, galactosamine and carbon tetrachloride induced hepatotoxicity in rats (Begam, M.*et al.,* 2007).

- ***Camellia sinensis (Theaceae)***

The leaves of *Camellia sinensis* are used to treat the cancer of duodenum, lung, liver and mammary gland. Catechins in combination with antioxidants with vitamin E are hypothesized to offer hepatoprotective defence against enzymes such as superoxide, dismutase and catalase (Haynes, R.H. et al., 1981).

- ***Cassia tora (Leguminosae)***

Cassia tora is used for medicinal purposes all over the world. Cassia alata, C. fistula and C. tora are the important species recommended for primary health care to treat ringworm and skin diseases. The isolated compounds rubrofusaruin, cassisoid were found to have hepato protective activity against galactosamine damage, which was higher than that of silybin (Gilman, K. et al.,1999).

- ***Cichorium intybus (Asteraceae)***

Cichorium intybus commonly known as Chicory is an indigenous perennial herb, well reputed ancient Indian medicine as a liver tonic. Accordingly it has been used as ayurvedic medicine for gall and liver disturbances and it forms an important component of several liver preparations in India(Kerri, A.M et al.,2005).

- ***Curcuma longa (Zingiberaceae)***

The hepatoprotective activity of the ethanol extract of *Curcuma longa* was studied against paracetamol-induced liver damage in rats (Srour, M. et al.,2000).

- ***Ginkgo biloba (Ginkgoaceae)***

Ginkgo biloba exhibits a variety of interesting pharmacological properties such as oxygen free radical scavenging activity, cyclonucleotidephosphodiesterase inhibition, membrane stabilizing effect, increase in blood fluidity and improvement in cognitive function. Among the various mechanisms involved, the antioxidant property is claimed to be one of the mechanisms of hepatoprotective effect (Scott, E.G. *et al.,* 1982).

In our study we are inducing hepatotoxicity by using ethanol and our aim is "to determine the hepatoprotective activity of a hydrodistilled extract of *Hemidesmus indicus* using ethanol induced rat model".

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1 LIVER (ANATOMY, HISTOLOGY & PHYSIOLOGY)

The liver is the heaviest gland of the body, weighing about 1.4 kg (about 3 lb) in an average adult. Among the organs of the body, it is second only to the skin in size. It is located inferior to the diaphragm and occupies most of the right hypochondriac and part of the epigastric regions of the abdominopelvic cavity[42].

2.2 ANATOMY [43]

Anatomy liver is the largest organ in the body weighing 1400-1600 gm in the males and 1200-1400 gm in females. There are 2 main anatomical lobes-right and left. The right being about six times the size of the left lobe. The right lobe has quadrate lobe on its inferior surface and a caudate lobe on the posterior surface. The right and left lobes are separated anteriorly by a fold of peritoneum called the falciform ligament, inferiorly by the fissure for the ligamentum teres and posteriorly by the fissure for the ligamentum venosum. The porta hepatis is the region on the inferior surface of the right lobe where blood vessels, lymphatic and common hepatic duct forms the hilum of the liver. A firm smooth layer of connective tissue called Glisson’s capsule encloses the liver and is continuous with the connective tissue of the porta hepatis forming a sheath around the structures in the porta hepatis. The liver has a double blood supply, the portal vein brings the venous blood from the intestine and spleen and the hepatic artery coming from the 21eliac axis supplies arterial blood to the liver. This dual blood supply provides sufficient protection against infarction in the liver. The portal vein and hepatic artery divide into branches to the right and left lobes in the porta. The right and left hepatic ducts also join in the porta to form the common hepatic duct. The venous drainage from the liver is into the right and left hepatic veins which enter the inferior vena cava. Lymphatics and the nerve fibres accompany the hepatic artery into their branching and terminate around the porta hepatis.

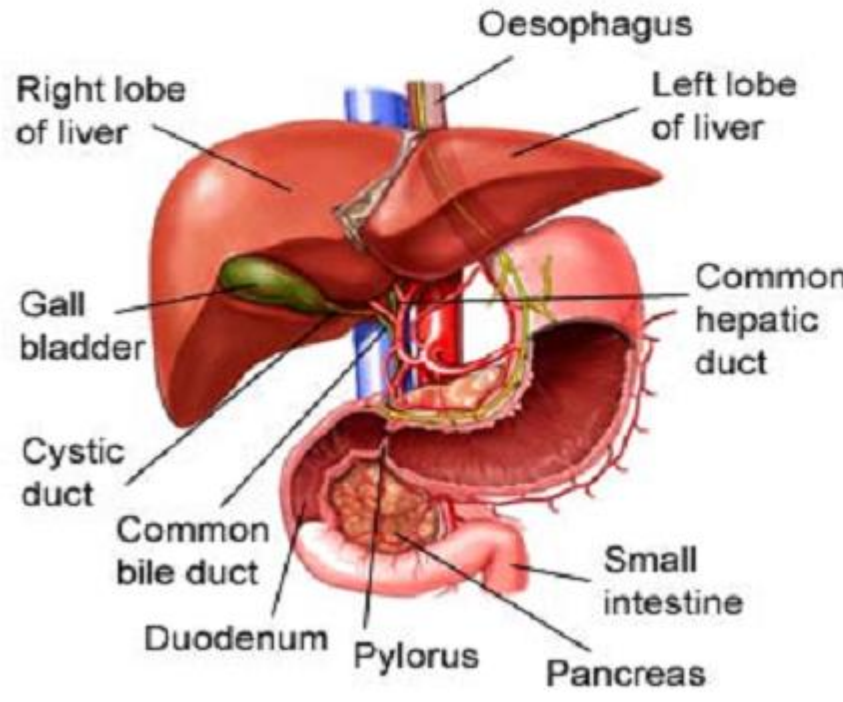


Fig. 4: Liver Anatomy.

2.3 HISTOLOGY[43]

The hepatic parenchyma is composed of numerous hexagonal or pyramidal classical lobules each with a diameter of 0.5 to 2 nm. Each classical lobule has a central tributary from the hepatic vein and at the periphery are 4 to 5 portal tracts or triads containing branches of bile duct, portal vein and hepatic artery. Cords of hepatocytes and blood containing sinusoids radiate from the central vein to the peripheral portal triads. However in most descriptions on pathology of the liver, the term lobule is used in its classical form. The blood supply to the liver parenchyma flows to the portal triads and to the central veins. Accordingly, the hepatic parenchyma of liver lobule is divided into 3 zones.

- Zone 1 or the periportal (peripheral) area is closed to the arterial and portal blood supply and hence bears the brunt of all forms of toxic injury.
- Zone 2 is the intermediate midzonal area.
- Zone 3 or the centrilobular area surrounds the central vein and is most remote from the blood supply and thus suffers from the effects of hypoxic injury.

The hepatocytes are polygonal cells with a round single nucleus and a prominent nucleolus. The liver cells have a remarkable capability to undergo mitosis and regeneration. Thus it is not uncommon to find liver cells containing more than

one nuclei and having polyploidy up to octoploidy. A hepatocyte has 3 surfaces; one facing the sinusoid and space of disse, the second facing the canaliculus and the third facing neighbouring hepatocytes. The blood-containing sinusoids between cords of hepatocytes are lined by discontinuous endothelial cells and scattered flat Kupffer cells belonging to the reticuloendothelial system. The space of disse is the space between hepatocytes and sinusoidal lining endothelial cells. A few scattered fat storing cells lie within the space of disse. The portal triad or tract besides containing portal vein radical, the hepatic arteriole and bile duct has a few mononuclear cells and a little connective tissue considered to be extension of Gilson's capsule. A limiting plate of hepatocytes surrounds the portal triads. The intrahepatic biliary system begins with the bile canaliculi interposed between the adjacent hepatocytes. The bile canaliculi are simply grooves between the contact surfaces of the liver cells and are convered by microvilli. These canaliculi join at the periphery of the lobule to drain eventually into terminal bile ducts or ductules which are lined by cuboidal epithelium. Hepatocytes perform several and very important roles in maintaining homeostasis and health. These functions include the synthesis of most essential serum proteins (albumin, carrier proteins, coagulation factors, many hormonal and growth factor) the production of bile and its carriers (bile acids, cholesterol, lecithin, phospholipids), the regulation of nutrients (glucose, glycogen, lipids, cholesterol, amino acids) and metabolism and conjugation of lipophilic compounds (bilirubin, anions, cations, drugs) for excretion in the bile or urine. Measurement of these activities to evaluate liver function is complicated by the multiplicity and variability of this functions[44].

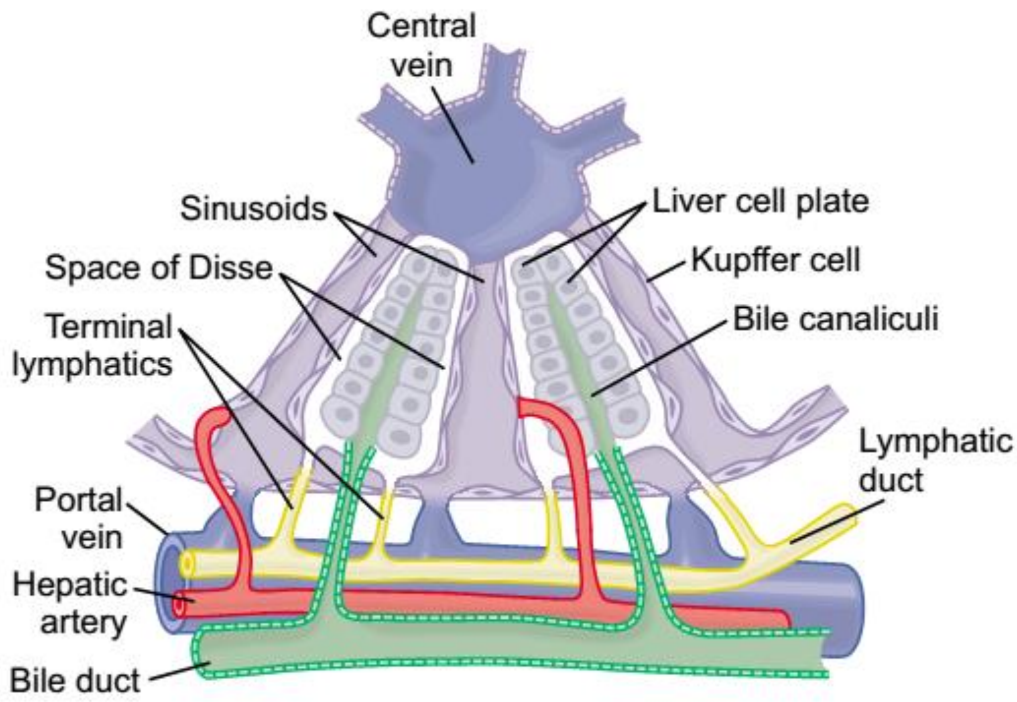


Fig. 5: Liver Histology.

The liver plays an astonishing array of vital functions for the maintenance, performance and regulating homeostasis of the body. It is involved with almost all the biochemical pathways to growth, fight against disease, nutrient supply, energy provision and reproduction (Sharma et al., 1991)[45].

The major functions of the liver are carbohydrate, protein and fat metabolism, detoxification, secretion of bile and storage of vitamin. Thus, to maintain a healthy liver is a very crucial factor for the overall health and well being (Subramaniam and Pushpangadan, 1999)[46].

More than 900 drugs have been implicated in causing liver injury and it is the most common reason for a drug to be withdrawn from the market. Some of the chemicals often cause subclinical injury to liver which manifests only as abnormal liver enzyme tests. Drug induced liver injury is responsible for 5% of all hospital admissions and 50% of all acute liver failures. More than 75 percent of cases of idiosyncratic drug reactions result in liver transplantation or death (Ostapowicz et al., 2002)[47].

2.4 LIVER FAILURE

This is a life-threatening condition that demands urgent medical care. Most often, liver failure happens gradually, over many years. It's the final stage of many liver

diseases. But a rare condition known as acute liver failure happens rapidly (in as little as 48 hours) and can be difficult to detect at first. Liver failure happens when large parts of the liver become damaged beyond repair and the liver can't work anymore.

There are two types of live failure:

- **Acute:** This is when your liver stops working within a matter of days or weeks. Most people who get this don't have any type liver disease or problem before this event.
- **Chronic:** Damage to your liver builds up over time and causes it to stop working.

Causes of Acute Liver Failure

The causes of acute liver failure, when the liver fails rapidly, include:

- **Acetaminophen overdose:** Large doses can damage your liver or lead to failure.
- **Viruses including hepatitis A, B, and E, the Epstein-Barr virus, cytomegalovirus, and herpes simplex virus:** They lead to liver damage or cirrhosis.
- **Reactions to certain prescription and herbal medications:** Some kill cells in your liver. Others damage the duct system that moves bile through it.
- **Eating poisonous wild mushrooms:** A kind called *Amanita phalloides*, also known as death cap, contains toxins that damage liver cells and lead to liver failure within a couple of days.
- **Autoimmune hepatitis:** As with viral hepatitis, this disease, in which your body attacks your liver, can lead to acute liver failure.
- **Wilson's disease:** This genetic disease prevents your body from removing copper. It builds up in, and damages, your liver.
- **Acute fatty liver of pregnancy:** In this rare condition, excess fat gathers on your liver and damages it.
- **Septic shock:** This overwhelming infection in your body can damage your liver or cause it to stop working.
- **Budd Chiari syndrome:** This rare disease narrows and blocks the bloodvessels in your liver.
- **Industrial toxins:** Many chemicals, including carbon tetrachloride, a cleaner and degreaser, can damage your liver.

Causes of Chronic Liver Failure

The most common causes of chronic liver failure include:

- **Hepatitis B:** It makes your liver swell and stops it from working the way it should.
- **Hepatitis C:** If you have it long-term, it can lead to cirrhosis.
- **Long-term alcoholconsumption:** It also leads to cirrhosis.
- **Hemochromatosis**: This inherited disorder causes your body to absorb and store too much iron. It can build up in your liver and cause cirrhosis.

2.5 HEPATOTOXICITY

The Damage or injury to the liver caused by a drug, chemical or other agent. Symptoms vary depending on the degree of exposure and hence extent of the liver damage or injury. Mild liver damage may cause few if any symptoms whereas severe damage can ultimately result in liver failure. More detailed information about the symptoms, causes, and treatments of Hepatotoxicity is available below.

Signs and Symptoms of Hepatotoxicity

The list of signs and symptoms mentioned in various sources for Hepatotoxicity are listed below:

- Nausea
- Vomiting
- Abdominal pain
- Loss of appetite
- Diarrhoea
- Tiredness
- Weakness
- Jaundice
- Yellow eyes
- Yellow skin
- Enlarged liver
- Abnormal liver function test results
- Swelling in feet
- Weight increase due to water retention
- Prolonged bleeding time

Hepatotoxicity Causes & Riskfactors

Experts believed that the main reason for having Hepatotoxicity is the consequence of

1. Exposure of poisons
2. Harmful agents
3. Toxic substances and
4. Biological hazards, etc. at any time of the patient's life.

It can also be brought about by,

1. Previous liver problems
2. Abdominal diseases and digestive diseases
3. Adverse reactions of *medications*
4. Toxicity
5. Poisoning and
6. Overdose from medications.

I will now try to elaborate Drug Induced Hepatotoxicity and different drug interactions that can cause Hepatotoxicity.

Hepatotoxic Drugs

There are drugs, substances, medications, or toxins that can be toxic to your liver that eventually when you use religiously may lead to Hepatotoxicity. It is always advisable that you ask your doctor of any prescribed drugs and medications for your illness management can be hepatotoxic. This comprises of over-the-counter drugs, prescription medications, food supplements, herbal remedies or alternative medicines. There are few examples of hepatotoxic drugs like Dacarbazine, DTIC-Dome, Sandimmune, Consupren and Sandimmun Neoral. But as I said a while ago it is very important to ask your doctor about every drug that you take.

Drug Interactions

Drug Interactions that can lead to Hepatotoxicity- It is not only hepatotoxic drugs that can cause hepatotoxicity, but some drugs when combined may react and in the end also lead to hepatotoxicity. These are only a few examples of drug combinations that can lead to liver toxicity:

a. INH and Tylenol
b. Isotamine and Tylenol
c. Laniazid and Tylenol
d. Nydrazid and Tylenol

This is why when you're taking drugs or medications you have to be very careful. Be truthful to your doctor on what medications are you taking when he will prescribe your medication. Ask your doctor about drug interactions, it is better to ask than regret it in the future that you have not done any precaution.

PLANT PROFILE

3. PLANT PROFILE

Figure 6: Whole *Hemidesmus indicus* Plant

Figure 7: ***Hemidesmus indicus***

Figure 8: Root of *Hemidesmus indicus*

Figure 9: Root of *Hemidesmus indicus*

Figure 10: Flowers of *Hemidesmus indicus*

Vernacular name(s) : Indian sarsaparilla, American sarsaparilla, Nannaari, Nannetti

Botanical name : *Hemidesmus indicus*

Family : Apocynaceae

3.1. Macroscopy:

The roots used in this study occur in pieces and about 3.95cm long and 0.21mm in diameter, cylindrical, thick and hard in nature. Externally brownish yellow in colour with transverse cracks and bark was easily detachable from the hard central core. Odour was pleasant and aromatic in nature.

3.2. Active constituents:

The roots of *Hemidesmus indicus* contain Hexatriacontane, Lupeol, its Octacosanoate,α-amyrin, β-amyrin, its Acetate and Sitosterol. It also contain Hemidesmin I, Hemidesmin II. Six pentacyclic triterpenes including two oleanenes, and three ursenes. The stem contains calogenin acetylcalogenin-3-0-β-D-digitoxopyrannosyl-0-β-D-digitoxopyronsyl-0-β-D-digitoxopyranoside. It also afforded 3-keto-lup-12-en-21 28-olide along with lupanone, lupeol-3-β-acetate, hexadecanoic acid, 4-methoxy-3-methoxybenzalaldehyde and 3-methoxy-4-5methoxybenzalaldehydglycosides-indicine and hemidine. The leaves contain tannins, flavonoids, hyperoside, rutin and coumarino[48].

Table 1. Structures of isolated compounds from *Hemidesmus indicus*

Compound name	Structure
Lupeol	
α-amyrin	
β-amyrin	
Triterpenes	
Tannins	
Flavonoids	

3.3. Traditional use:

Apart from hepatoprotective activity *Hemidesmus indicus* is used to make beverages and also used in traditional medicine. In Ayurveda it goes by the name of *Ananthamoola*, also known locally in Southern India as *Naruneendi* or *Nannari*, (Sanskrit meaning: *endless root*). It is also known as *Anant Vel* /*Maeen Mool* in Marathi. In southern states of India (particularly Tamil Nadu), sarsaparilla roots are called *Maahali*or *Mahani Kizhangu* and in its pickled form is also served along with rice dishes. It is also called the false sarsaparilla. It is administered in the form of powder, infusion or decoction as syrup. It is one of the Rasayana plants of Ayurveda. It is sometimes confused with another Ayurvedic herb called white *sariva*. The alcoholic extract of *Hemidesmus indicus* R.Br. possesses a significant antinociceptive activity[49]. The extracts from the root are used in syrup with sugar and a dash of lemon (Sharbat) and served at most small refreshment shops in South India.

Table no: 2. Reported activities of *Hemidesmus indicus*

Fraction used	Dose	Parameters measured	Use
Aqueous extract	0.04mg/kg to 0.08mg/kg	Inhibit growth of S.aureus, K.pneumoniae, P.aeruginosa.	Antimicrobial activity
Aqueous and methanolic root extract encapsulated poly lactide-co-glycolide (Ravikiran T .et al.,2016 Apr.)	50mg	DPPH, Superoxide and Hydroxyl radical scavenging assays.	Antioxidant activity of *Hemidesmus indicus.*

Aqueous methanolic root extract (Sarada D et al.,2018,Feb 6)	1mg	Tumour necrosis factor alpha(TNFα) secretion by ELISA, translocation of nuclear factor kappaB(NF-κB)	The alcoholic water extract of *Hemidesmus indicus* shows anti-inflammatory and antihyperlipidemic activities.
Methanolic extract (Lakshman et al, 2005 Dec) [50].	100,200, 400mg/kg	Carrageenan-induced rat paw oedema and brewer's yeast-induced pyrexia in rat.	Antipyretic activity of *Hemidesmus indicus*.
Hydroalcoholic root extract (Farook et al.,2011)	300mg/kg	Yeast induced rat.	Decrease in body temperature.

AIM & OBJECTIVES

4. AIM & OBJECTIVE

4.1 AIM: To evaluate hepatoprotective activity of *Hemidesmus indicus.* In Ethanol induced hepatotoxicity.

4.2 OBJECTIVE:

1. To obtain the hydro distillated root extract from *Hemidesmus indicus*.
2. To find out the biochemical parameters.
3. To determine the level of GSH, SOD and CAT in rats with hepatotoxicity.
4. To study the morphological changes in liver cells.

PLAN OF STUDY

5. PLAN OF STUDY

5.1 Phase I – Plant profile

- Collection of the plant flower *Hemidesmus indicus*
- Aqueous extraction of *Hemidesmus indicus* (Hydrodistillation)
- Qualitative phytochemical test

5.2 Phase II – Treatment protocol

- Drug treatment - 28 days
- Induction of hepatotoxicity using Ethanol (100mg/kg) for 28 days

5.3 Phase III – Biochemical estimations in plasma.

1. Serum glutamate pyruvate transaminase (SGPT/ALT)
2. Serum glutamate oxaloacetate transaminase (SGOT/AST)
3. Serum alkaline phosphatase (SALP)
4. Serum bilirubin (direct and total)
5. Serum total proteins

5.4 Phase IV – Evaluation of antioxidant enzymes in liver.

1) Glutathione (GSH)
2) Lipid peroxidation (LPO)
3) Catalase (CAT)
4) Super oxide dismutase (SOD)

5.5 Phase V – Histopathology

- Liver.

MATERIALS & METHODS

6. MATERIALS AND METHODS

TABLE NO: 3 List of chemicals used

Chemicals	Manufacturers
Ethanol	CHANGSHU YANGYUANCHEMICALS
ALAT kit	AGAPPE Merck ltd,India
ASAT kit	AGAPPE Merck ltd,India
ALP kit	AGAPPE Merck ltd,India
Cholesterol kit	AGAPPE Merck ltd,India
Triglycerides kit	AGAPPE Merck ltd,India
Creatinine kit	AGAPPE Merck ltd,India
Bilirubin kit	AGAPPE Merck ltd,India
Silymarin	Sigma Aldrich, USA
1,1,3,3-tetraethoxy propane	Sigma Aldrich, USA
5,5'-Dithio-Bis (2-Nitrobenzoic Acid)	Himedia lab limited, India
Glutathione reductase	Sigma Aldrich, USA
Hydrogen peroxide	Merck limited, India
NADH	Himedia lab limited, India
NADPH	SRL limited, India
Nitro blue tetrazolium	Himedia lab limited, India
Phenazine metho sulphate	SRL limited, India
N-hexane	Himedia lab limited, India
Sodium dihydrogen orthophosphate	Himedia lab limited, India
Super oxide dismutase	Sigma Aldrich, USA

6.1 Methodology

Experimental animals

Healthy Wistar albino rats of either sex weighing 150-200 g were used. Animals used in the study were procured from registered breeder. The animal care and handling was carried out according to CPCSEA guidelines. Animals were acclimatized to the animal quarantine for one week prior to the experiment under controlled conditions of temperature (27 ± 2ºC) and were housed in sterile polypropylene cages containing paddy husk as bedding material with maximum of six animals in each cage. The rats were fed on standard food pellets and water *adlibitum*. The experimental protocols were approved by the Institutional Animal ethical Committee of RVS and experiments were performed according to the CPCSEA guidelines.

Collection of plant material and preparation of extract:

The roots of *Hemidesmus indicus* used for the present studies were collected from Calicut, Kerala, in April 2019 and authenticated. The collected roots material were cleaned to remove the adhered dust particles and were then shade dried. The dried plant materials were coarsely powdered, weighed and stored in an air tight container till use. The coarse powder was packed into Soxhlet column and extracted with 70% ethanol for about 48 h[51]. The solvent was evaporated using rotary flash evaporator to get syrupy consistency. Then the dried extract was stored in air tight container in refrigerator below 10°C.

6.2. PRELIMINARY PHYTOCHEMICAL SCREENING[52,53]

Preliminary phytochemical screening was carried out for hydroalcoholic extract of *Hemidesmus indicus* roots as described below.

A. Tests For Alkaloids

0.5gm extract was dissolved in 10 ml of dilute hydrochloric acid and filtered. The filtrate was used to test the presence of alkaloids:

I. Mayer’s test

To one ml of filtrate, 2 ml of Mayer’s reagent was added in a test tube. Formation of Yellow cream precipitate indicates the presence of alkaloids.

II. Wagner’s test

One ml of filtrate was treated with few drops of Wagner’s reagent. Formation of reddish brown precipitate indicates the presence of alkaloids.

III.Dragendorff's test

One ml of filtrate was treated with few drops of Dragendorff's reagent. Formation of reddish brown precipitate indicates the presence of alkaloids.

IV.Hager's test:

One ml filtrate was treated with few drops of Hager's reagent. Formation of yellow precipitate indicates the presence of alkaloids.

B. Tests for glycosides

I.Bromine water test:

Test extract was dissolved in bromine water. Formation of yellow precipitate indicates the presence of glycosides.

II.Baljet test:

Test extract was treated with sodium pirate. Formation of yellow to orange colour indicates the presence of glycosides.

III.Keller-Killiani test:

0.5g of dried extract was dissolved in 2 ml of glacial acetic acid containing one drop of ferric chloride solutions. This was then under laid with 1 ml of concentrated H_2SO_4. It forms two layers, lower layer reddish brown and upper acetic acid layer turns bluish green, indicates the presence of glycosides.

IV.Legal's test:

Test extract was treated with pyridine (made alkaline by adding sodium nitroprusside solution). Formation of pink to red colour indicates the presence of glycosides.

C. Tests For Tannins

I.Ferric chloride test:

Few drops of 5% w/v FeCl3 solution was added to 1-2ml of the extract. Formation of brown colour indicates the presence of pseudo tannins.

II.Vanillin hydrochloride test:

Extract was treated with vanillin hydrochloride reagent. Formation of purplish red colour indicates the presence of tannins.

III.Gelatin test:

Extract was treated with gelatine solution. Formation of white precipitate indicates the presence of tannins.

D. Tests For Saponins

I.Sulphur test:

Sulphur was added to the extract solution. Sulphur sinks at bottom indicates the presence of saponins.

II.Froth’s test:

The extract was diluted with distilled water and shaken for 15 min. Formation of foam indicates the presence of saponins.

III.Liberman Buchard’s test:

The extract was treated with chloroform and filtered. The filtrates were treated with few drops of acetic anhydride boiled and cooled. Concentrated sulphuric acid was added through the sides of the test tube. The formation of brown ring at the junction indicated the presence of steroidal saponins.

E. **Tests For Carbohydrates**

Extracts were dissolved individually in 5ml of distilled water and filtered. The filtrates were used to test the presence of carbohydrates.

I.Molisch’s Test:

Filtrates were treated with 2 drops of alcoholic α-naphthol solution in a test tube and 2 ml concentrated sulphuric acid was added carefully along the sides of the test tubes. Formation of violet ring at the junction indicates the presence of carbohydrates.

II.Benedict’s Test:

Filtrates were treated with Benedict’s reagent and heated on water bath. Formation of an orange red precipitate indicated the presence of reducing sugars.

III.Fehling’s Test:

Filtrates were hydrolyzed with dilute hydrochloric acids, neutralized with alkali and heated with Fehling’s A and B solutions. Formation of red precipitate indicates the presence of reducing sugars.

IV.Barfoed’s test:

1ml Barfoed’s reagent was added to 1ml of extract and heat for 2 min. Formation of red precipitate indicates the presence of carbohydrate.

V.Seliwanoff’s test:

Plant Extract was treated with Seliwanoff’s reagent and heat strongly. Formation of a characteristic cherry red colour indicates the presence of keto sugar.

F. Tests For Flavonoids

I.Lead acetate test:

Lead acetate solution was added to small amount of extract. Formation of yellow precipitate indicates the presence of flavonoids.

II.Shinoda test:

A little quantity of extract was dissolved in alcohol with few fragments of Mg turnings and con: HCl drop wise. Formation of pink or crimson-red colour indicates the presence of flavonoids.

III.Alkaline reagent test:

Increasing amount of sodium hydroxide was added to the sample extract. Formation of yellow colouration observed which disappears upon addition of acid indicates the presence of flavonoids.

IV.Ferric chloride test:

Extract was treated with ferric chloride solution. Formation of Intense green to black colour indicates the presence of flavonoids.

G. **Tests For Steroids**

I.Salkowski reaction:

2mg of dry extract was shaken with $CHCl_3$, to the $CHCl_3$ layer, H_2SO4 was added slowly by the sides of test tube. Formation of red colour indicates the presence of steroids.

II.Lieberman Burchard's test:

2mg of dry extract was dissolved in acetic anhydride, heated to boiling, cooled and then 1ml of conc. H_2SO_4. Formation of red violet or green colour indicates the presence of Steroids.

The hydro alcoholic extracts of *Hemidesmus indicus* roots were subjected to the following investigations:

6.3. PHARMACOLOGICAL ACTIVITIES

1. Determination of acute toxicity (LD_{50})
2. Hepatoprotective activity

1. Determination of Acute toxicity (LD_{50})[54]

Preparation of dose:

The hydro alcoholic extract of the leaves of *Hemidesmus indicus* was suspended in Tween-80 to prepare a dose of 2000 mg/kg b.w. of animal and administered 1ml/100 g b.w. of the animal.

Acute toxicity study of the extract was done according to acute toxic classic method (OECD guideline 425, 2006) using albino female rats to determine the safe dose.

Procedure

The animals were kept fasting for overnight with sufficient water. The suspension of extract, prepared in Tween-80, was administered orally for one animal at the limit dose of 2000 mg/kg and was observed for 14 days (with special attention for the first 4 h of administration followed by next 20 h) for mortality, general behavior and signs of discomfort. If the animal survived, another four animals were dosed sequentially so that total five animals were tested.

The following check list was employed for the acute toxicity studies:

i.Behavioural Profile:

Awareness: Alertness, stereotypy, visual placing.

Mood: Grooming, restlessness, fearfulness.

ii.Neurological profile:

Motor activity: Spontaneous activity, touch response, pain response, tremor, grip strength, corneal reflex.

iii.Autonomic profile:

Writhing, defecation, urination, piloerection.

2. **Evaluation of hepatoprotective activity**

6.4. GROUPING OF ANIMALS:

Animals were divided into five groups, each group containing six animals.

Table 4; Grouping of animals

Group	**Treatment**
I	CONTROL
II	ETHANOL.0.5ML/100G)P.O
III	SILYMARIN 100mg/kg /+ Ethanol/p.o
IV	HI 200 mg/kg /p.o + Ethanol 0.5ml/100g
V	HI 400 mg/kg/p.o + Ethanol 0.5ml/100g

6.5. TREATMENT PROTOCOL:

All the extract treatments were given orally for 28 days. The experiment duration was 4 weeks. All animals were divided into 5 groups: control (received distilled water as drinking source), Ethanol control (0.5mL C2H5OH/100 gm body weight),Stantard Silymarin+ethanol (100 mg/kg+0.5mL C2H5OH) , ethanol + HI (0.5mL C2H5OH + 200 mg HI/1.Kg body weight), and ethanol + HI (0.5mL C2H5OH + 400mg HDTP/ 1.Kg body weight). The animals of alcohol control group were administered oral dose of ethanol everyday between 10:00 AM and 11:00 AM. Experimental animals of group 4 and group 5 were given orally 200mg and 400mg/1.kg body weight dose of HI, respectively, after 1 hour of feeding of alcohol.

6.6. BLOOD COLLECTION

On 28th day blood was collected through retro orbital puncture and analyzed for various biochemical parameters. Blood was allowed to clot at room temperature for 30 min, subjected to centrifugation (3000 rpm for 15 min.) and estimation of biochemical parameters.

6.7. PREPARATION OF LIVER SAMPLES

After blood collection, rats were killed by cervical dislocation and livers were dissected out immediately, washed with ice cold saline, cleaned and weighed. Small pieces of liver were cut and transferred into 10 % neutral formalin solution for histological studies. Other portions of liver washed with ice cold saline and homogenized in 10 % (w/v) ice cold phosphate buffer (0.1 M pH 7.4) and centrifugation at 10,000 rpm for 15 min at 4°C. The supernatants obtained were kept in deep freezer at 20°C until used for the assays of SOD.

Biochemical parameters estimated:

1. Serum alkaline phosphatase (ALP)
2. Serum glutamate pyruvate transaminase (SGPT)
3. Serum oxaloacetate transaminase (SGOT)
4. Serum total bilirubin (BILT)
5. Serum direct bilirubin (BILD)
6. Serum total protein

Assay of endogenous antioxidant parameters:

1. Superoxide dismutase (SOD)
2. Reduced glutathione (GSH)
3. Lipid peroxidation (MDA)

4. Catalase (CAT)

6.8. Methods for estimation of biomarkers

The biochemical parameters were estimated as per the standard procedure prescribed by the manufacturer's instruction manual provided in the kit using semi auto analyzer.

1. Estimation of ALP[55]

Principle:

Serum alkaline phosphatase hydrolyses p-nitrophenyl phosphate into pnitrophenol and phosphate in the presence of oxidizing agent Mg^{2+}. This reaction is measured as absorbance is proportional to the ALP activity.

Reaction:

$$\text{P-Nitro phenyl phosphate} + \text{H2O} \xrightarrow{\text{ALP, } Mg^{+2}} \text{p- nitro phenol} + \text{Phosphate}$$

ALP: Alkaline phosphatase.

The increase in absorbance due to formation of 4- nitro phenolate is measured photo metrically and is directly proportional to ALP activity in sample.

Procedure

Pipette	Sample
Working reagent	1000µl
Sample	20µl

Mix and incubate for 1 min at 37°C. Read the change in absorbance per 20 sec, during 1 min.

2. Estimation of Serum Alanine Transaminase (U.V. kinetic) (ALT or SGPT)[56]

Principle

Alanine amino transferase catalyses the transfer of amino group from alanine to α-keto glutarate (α-KG) with the formation of Glutamate and Pyruvate. The liberated Pyruvate reduced to lactate, by lactate dehydrogenase (LDH) in the same reaction an equivalent amount of NADH is oxidized to NAD.

Reactions

$$\text{L-Alanine} + \alpha\text{-keto glutarate} \xrightarrow{\text{GPT}} \text{Pyruvate} + \text{L-glutamate}$$

LDH

Pyruvate + NADH + H^+ ⟶ Lactate + NAD^+

Procedure:

Pipette	**Sample**
Working reagent	1000µl
Sample	100µl

Mix and incubate for 1 min at 37°C read the change in absorbance per 20 sec during 1min using semi auto analyzer.

3. Estimation of Serum Aspartate Amino Transaminase (U.V. kinetic) (AST or SGOT)[57,58]

Principle:

SGOT catalyses the transfer of amino group from L- Aspartate to α- Keto glutarate with the formation of oxaloacetate and L-glutamate. The rate is monitored by an indicator reaction coupled with malate dehydragenase (MDH) in which the oxaloacetate formed is converted to malate ion in the presence of NADH (nicotinamide adenine dinucleotide). The oxidation of NADH in this reaction is measured as a decreasing in the absorbance of NADH at 340 nm, which is proportional to SGOT activity.

Reactions

SGOT or AST

L- Aspartate + α- Ketoglutarate ⟶ Oxaloacetate + L glutamate

MDH

Oxaloacetate + NADH + H^+ ⟶ L- Malate + NAD^+

Where: - AST: Aspartate amino transferase, MDH: malate dehydrogenase

LDH: Lactate dehydrogenase

Procedure:

Pipette	**Sample**
Working reagent	1000µl
Sample	100µl

Mix and incubate for 1 min at 37°C read the change in absorbance per 20 sec, (ΔOD/20sec) during 1 min using semi auto analyzer.

4. Estimation of Serum Bilirubin[59]

Bilirubin reacts with diazotized sulphanilic acid in acidic medium to form a pink coloured azobilirubin with absorbance directly proportional to bilirubin concentration. Direct bilirubin, being water soluble directly reacted in acidic medium. However, indirect and unconjugated bilirubin is solubilised using a surfactant and then it reacts similar to direct bilirubin.

Procedure

	Total bilirubin		**Direct bilirubin**	
	Sample blank	**Test**	**Sample blank**	**test**
Total bilirubin reagent	1000µL	1000µL	---	---
Direct bilirubin reagent	---	---	1000µL	1000µL
Activator total / direct	--	20µL	---	20µL
Serum	50µl	50µl	50µl	50µl

Mix well and incubate for exactly 5min. measure the absorbance of the sample against respective sample blank, using semi auto analyzer.

5. Estimation of Serum Total Proteins [60]

Principle

Serum proteins together with copper ions form a violet colour complex in alkaline solution. The absorbance of the colour is directly proportional to the concentrations.

Reaction:

$$\text{Proteins} + Cu^{++} \longrightarrow \text{Coloured complex}$$

Procedure

Addition sequence	**Blank**	**Standard**	**Test**
Working reagent	1000µl	1000µl	1000µl
Standard	-	20 µl	-
Sample	-	-	20 µl

Mix and incubate for 10 min at 37°c. Measure the absorbance of standard and sample against reagent blank, using semi auto analyzer.

6.9. *In-vivo* ANTIOXIDANT ACTIVITY

METHODS FOR ESTIMATION OF OXIDATIVE STRESS:

1. Estimation of lipid peroxidation[61]

Principle:

Malondialdehyde, formed from the breakdown of polyunsaturated fatty acids, serves a a convenient index for determining the extent of peroxidation reaction. Malondialdehyde reacts with thiobarbituric acid to form red color species (TBARS), which is measured at 535 nm.

Reagents:

TBA-TCA-HCl reagent: [15% w/v TCA, 0.375% w/v TBA and 0.2 ml of 0.25 N HCl] this solution was mildly heated to assist the dissolution of TBA.

Procedure:

- 1 ml of liver homogenate was combined with 2ml of TCA-TBA-HCl reagent and mixed thoroughly
- The solution was heated for 15 min in a boiling water bath. After cooling, the flocculent precipitate was removed by centrifugation at 1000 rpm for 10 min
- The absorbance of the supernatant was measured at 532 nm against a blank that contains all the reagents minus the liver homogenate.

Malondialdehyde concentration (M) = Absorbance at 532 nm / 1.56 x 10

2. Estimation of superoxide dismutase (SOD) [62]

SOD was assayed according to the method of Kakkar et al.(1984)

PRINCIPLE

The assay of SOD is based on the inhibition of theformation of NADH-phenazine methosulphate-nitroblue tetrazolium formazon. The colour formed at the end of the reaction can be extracted into butanol and measuredat 560nm.

REAGENTS

1. Sodium pyrophosphate buffer (0.025M, pH 8.3)
2. Phenazine methosulphate (PMS) (186µM)
3. Nitroblue tetrazolium (NBT) (300µM)
4. NADH (780µM)
5. Glacial acetic acid

6. n-butanol
7. Potassium phosphate buffer (50mM, pH 6.4)

PROCEDURE

The assay mixture contained 1.2ml of sodium pyrophosphate buffer, 0.1ml of PMS, 0.3ml of NBT, 0.2ml of the liver homogenate (supernant) and water in a total volume of 2.8ml. The reaction was initiated by the addition of 0.2ml of NADH. The mixture was incubated at 30 °C for 90 seconds and arrested by the addition of 1.0ml of glacial acetic acid. The reaction mixture was then shaken with 4.0ml of n-butanol, allowed to stand for 10 minutes and centrifuged. The intensity of the chromogen in the butanol layer was measured at 560nm in a spectrophotometer.

3. Estimation of reduced glutathione (GSH)[63]

Reduced glutathione was determined by the method of Moron et al. (1979).

PRINCIPLE

Reduced glutathione on reaction with DTNB (5,5'-dithiobis nitro benzoic acid) produces a yellow coloured product that absorbs at 412nm

REAGENTS

1. TCA (5%)
2. Phosphate buffer (0.2M, pH 8.0)
3. DTNB (0.6mM in 0.2M phosphate buffer)
4. Standard GSH (10nmoles/ml of 5% TCA)

PROCEDURE

Sliced liver was homogenized with 10 ml of 10% TCA in ice cold condition and centrifuged at 3000 rpm for 10 minutes. The supernatant (0.1ml) was made up to 1.0ml with 0.2M sodium phosphate buffer (pH 8.0). Two ml of freshly prepared DTNB solution was added and the intensity of the yellow color developed was measured in a spectrophotometer at 412nm after 10 minutes. The values are expressed as nmoles GSH/g sample.

4. Estimation of catalase (CAT) [64]

The liver was homogenized in 0.01 M phosphate buffer (pH 7.0) and centrifuge at 5000 rpm. The reaction mixture consisted of 0.4 ml of hydrogen peroxide (0.2 M), 1 ml of 0.01 M phosphate buffer (pH 7.0) and 0.1 ml of liver homogenate (10% w/v). The reaction of the mixture was stopped by adding 2 ml of dichromate-acetic acid reagent (5% K2Cr2O7 prepared in glacial acetic acid). The changes in the absorbance was measured at 620 nm and recorded. Percentage

inhibition was calculated using the equation:

% Increase in CAT = (Abs of control- Abs of test / Abs of control) x 100

6.10.HISTOPATHOLOGICAL STUDIES:

Tissue Fixation

Liver tissues of rat was removed and washed with normal saline.

Formaldehyde, as 4% buffered formaldehyde (10% buffered formalin), is the most widely employed universal fixative particularly for routine paraffin embedded sections. The cleared tissue was fixed in 10% natural buffered formalin solution (pH 7.0 - 7.2).

Statistical analysis

All data were expressed as mean ±SEM. The statistical significance between groups was compared using one way ANOVA, followed by Dunnett's (multiple comparison test). The analysis was carried out by using Graph pad prism software of version 5.03.

RESULTS

7. RESULTS

7.1 EXTRACTION OF PLANT MATERIAL

The percentage yield of *Hemidesmusindicus* was found to be 12. 72% w/w as shown in Table No. 5.

Table No. 5: Percentage yield of crude extract of *Hemidesmus indicus* root.

Solvent	Color	Percentage yield
Hydroalcoholic	Dark brown	12. 72%

Preliminary phytochemical screening:

Preliminary phytochemical analysis of extract is shown in **Table No. 6** revealed the presence of following phytochemicals: Carbohydrates, Flavonoids, Glycosides, Saponins, Tannins and Steroids.

Table No. 6: Preliminary phytochemical screening.

SL. No	Test	Reagent	Result
1	Alkaloids	Mayer's reagent	-ve
2	Carbohydrates	Barfoed's reagent	+ve
3	Steroids	Con.Sulphuric acid	+ve
4	Cardiac Glycosides	Glacial acetic acid + $FeCl_3$+Con. Sulphuric acid	+ve
5	Saponins	Water + Shake	+ve
6	Tannins	Lead acetate	+ve
7	Flavonoids	Alikaline reagent	+ve
8	Phenolic Compounds	Alcohol + $FeCl_3$	+ve
9	Aminoacids	Ninhydrin reagent	+ve

(+ = Present in test, - = Absence in test)

7.2 ACUTE TOXICITY STUDY (LD_{50})

Hydroalcoholic extract of *Hemidesmus indicus* root was studied for acute toxicity at the dose level of 2000 mg/kg, p.o. according to OECD guideline Annexure 425. It was found to be safe up to 2000 mg/kg body wt. by oral route. There was no mortality and no signs of toxicity and extract were found to be safe. So two dose levels i.e., 200 mg/kg ($1/10^{th}$), and 400 mg/kg ($1/5^{th}$) body weight were selected for the present study.

7.3 PHARMACOLOGICAL ACTIVITIES

Evaluation of Hepatoprotective Activity of HI on ETHANOL Induced Hepatic Damage in Rats

In the present study, the hepatotoxicity was successfully produced by administration of ETHANOL.0.5ML/100G)P.O. for alternative days and the hepatoprotective activity of HI was determined from the serum parameters SGOT, SGPT, TP, TB, DB and ALP. The results were further confirmed by evaluation of liver endogenous antioxidant enzymes of the rats.

Biochemical parameters

The effect of extracts of *Hemidesmus indicus* root on various biochemical parameters are shown in Table No. 7 and Fig. 11, 12, 13. It was observed that, ethanol administration resulted in a significant elevation in the levels of SGOT, SGPT, TB, DB, ALP and decrease in level of TP compared to that of control group. The standard (Silymarin) treatment showed extremely significant ($P<0.001$) reduction in SGPT, SGOT, ALP, BILT & BILD and extremely significant ($P<0.001$) increase in total protein. It was observed that treatment with HI at 200 and 400 mg/kg, b.wt groups restored the activities of SGOT, SGPT, DB, TB, ALP and TP. HI-200 treated animals showed extremely significant ($P<0.001$) reduction in ALP & SGPT levels and extremely significant ($P<0.001$) increase in total protein, moderately significant ($P<0.01$) in SGOT, DB & TB level. HI-400 treated animals showed extremely significant ($P<0.001$) reduction in SGOT, ALP & SGPT levels and extremely significant ($P<0.001$) increase in Total protein, moderately significant ($P<0.01$) reduction in DB & TB level as compared to toxic control group.

Table No. 7: Effect of HI on biochemical parameters in Ethanol induced liver damage in rats

Groups	Treatment	ALP (U/l)	SGOT (U/l)	SGPT (U/l)	TB (mg/dl)	DB (mg/dl)	T.P (g/dl)
Normal control	Saline	135.2± 3.23	85.56± 3.01	58.51± 1.33	0.915± 0.012	0.23± 0.009	8.06± 0.06
Toxic control	ETHANOL.0. 5ML/100G)P. O	358.4± 7.03	175.4± 7.43	134.3± 2.24	2.648± 0.018	0.51± 0.008	4.63± 0.12
Standard	Silymarin 100mg/kg p.o	177.4± 5.84***	97.32± 2.06***	76.34± 2.46***	1.120± 0.01***	0.27± 0.008***	7.70± 0.07***
Low dose	HI 200 mg/kg p.o	301.6± 4.24***	151.8± 1.91**	106.8± 1.99***	2.817± 0.005**	0.50± 0.006**	6.68± 0.067***
High dose	HI 400 mg/kg p.o	251.6± 4.26***	106.5± 3.07***	96.60± 2.93***	2.808± 0.006**	0.49± 0.005**	7.28± 0.073***

One way ANOVA followed by Dunnett's t test. All the values are Mean±SEM , n=6. [a]$p < 0.001$ when compared with vehicle treated control group with toxic control. ns, $p > 0.05$, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ when compared with toxic control.

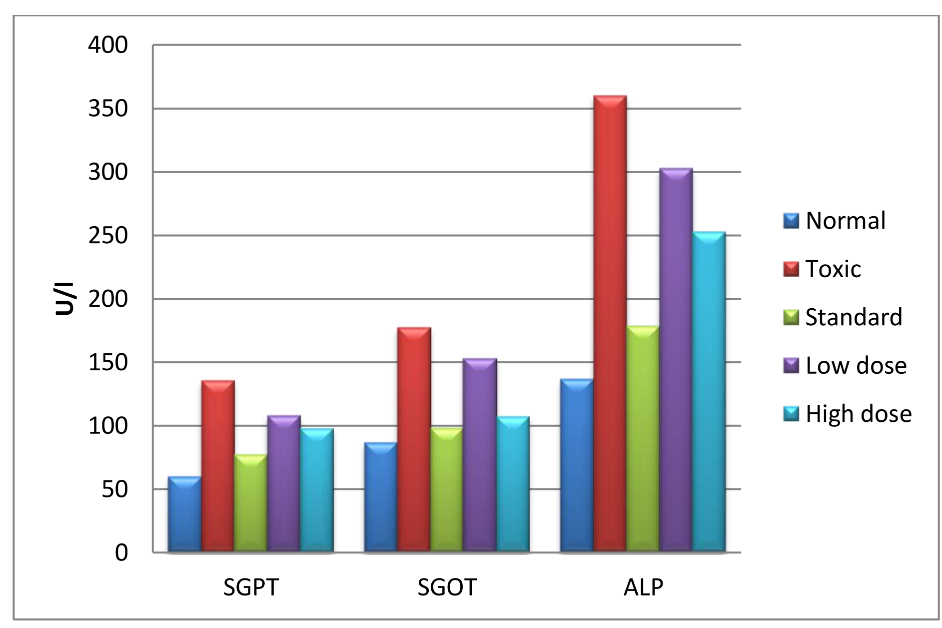


Fig.11: Effect of Silymarin and HI on Serum SGPT,SGOT & ALP in Ethanol induced liver toxicity.

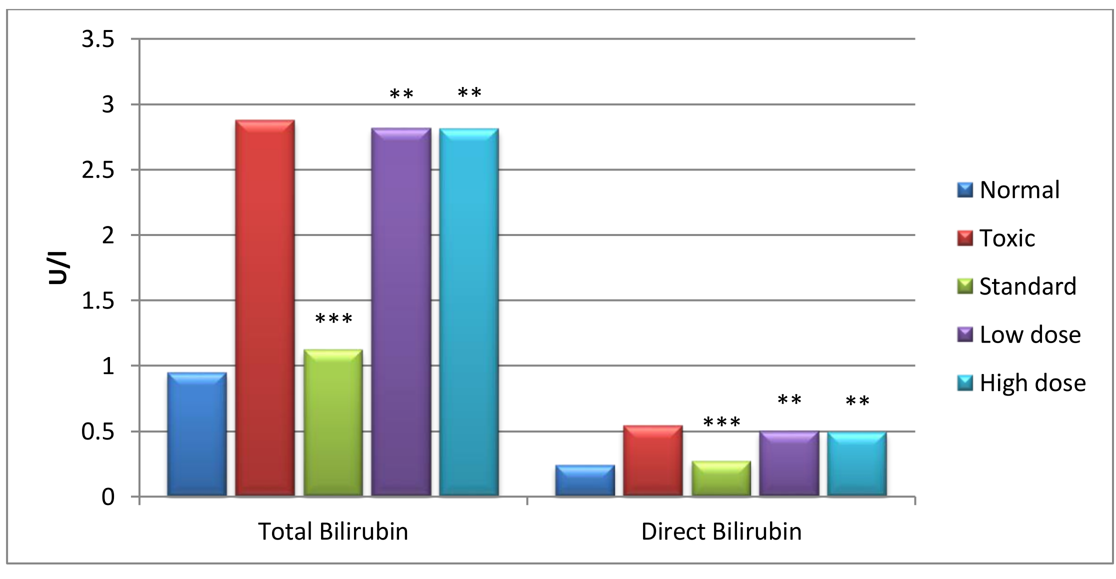


Fig.12: Effect of Silymarin and HI on Serum DB & TB in Ethanol induced liver toxicity.

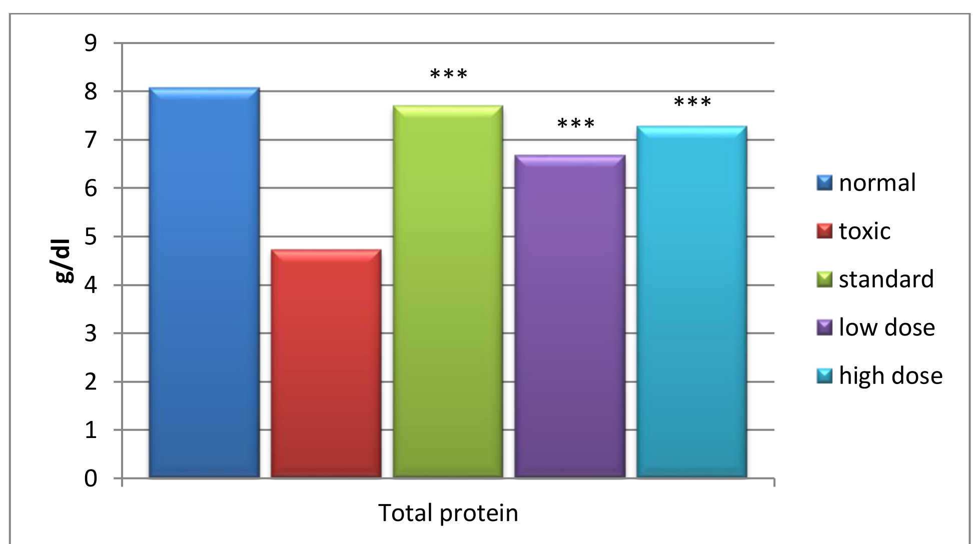


Fig.13: Effect of Silymarin and HI on Total protein in Ethanol induced liver toxicity.

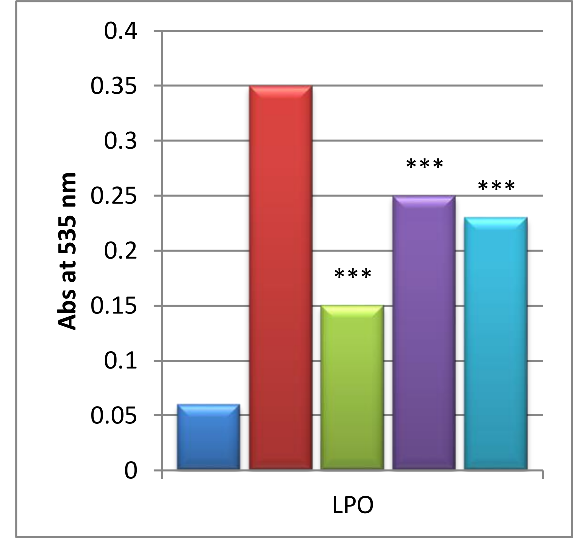
1. Evaluation of liver endogenous antioxidant enzymes

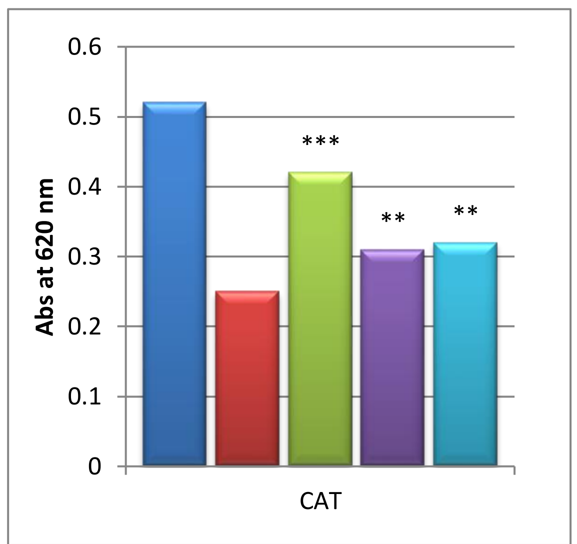
Table No. 8 and Fig.14 shows the effects of extracts of HI on LPO, SOD, GSH and CAT concentrations in rat liver after challenging with Ethanol. It was observed that animals treated with Ethanol developed a hepatic damage, increase in LPO and decrease in GSH, CAT & SOD when compared to normal control. Animals treated with standard (Silymarin) showed extremely significant (P<0.001) increase in GSH, CAT & SOD and decrease in LPO.

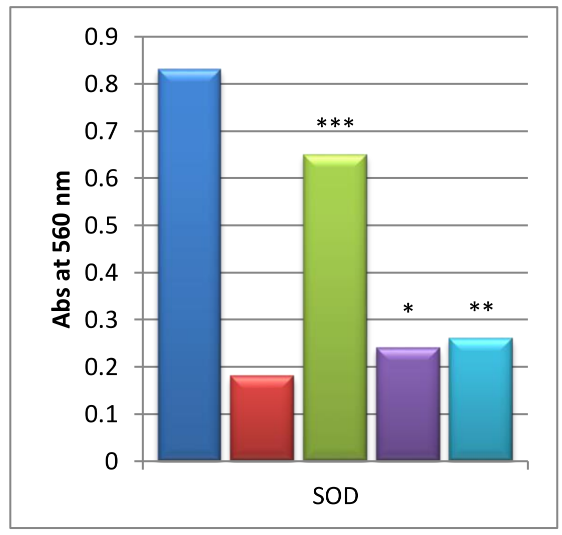
HI-200 treated animals showed extremely significant (P<0.001) decrease in LPO, moderately significant (P<0.01) increase in GSH & CAT and less significant (*p<0.05) increase in SOD. HI-400 treated animals showed extremely significant (P<0.001) decrease in LPO, extremely significant (P<0.001) increase in GSH and moderately significant (P<0.01) increase in SOD & CAT.

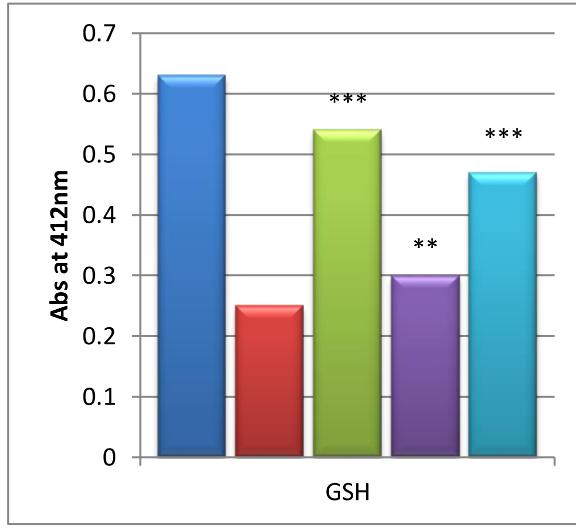
Table No. 8: Effect of Silymarin and HI on LPO, SOD, GSH, and CAT in Ethanol induced liver toxicity

Groups	Treatment	LPO (Abs at 535 nm)	SOD (Abs at 560 nm)	GSH (Abs at 412nm)	CAT (Abs at 620 nm)
Normal control	Saline	0.06± 0.009	0.83± 0.009	0.63± 0.009	0.52± 0.013
Toxic control	ETHANOL .0.5ML/100 G)P.O	0.35± .007	0.18± 0.008	0.25± 0.009	0.25± 0.013
Standard	Silymarin 100mg/kg p.o	0.15± 0.009***	0.65± 0.02***	0.54± 0.011***	0.42± 0.015***
Low dose	HI 200mg/kg p.o	0.25± 0.007***	0.45± 0.007*	0.30± 0.013**	0.31± 0.008**
High dose	HI 400mg/kg p.o	0.23± 0.008***	0.62± 0.006**	0.47± 0.010***	0.41± 0.011**
One way ANOVA followed by Dunnett's t test. All the values are Mean±SEM , n=6. [a]p< 0.001 when compared with vehicle treated control group with toxic control. ns, p>0.05, *p<0.05, **p<0.01, ***p<0.001 when compared with toxic control.					









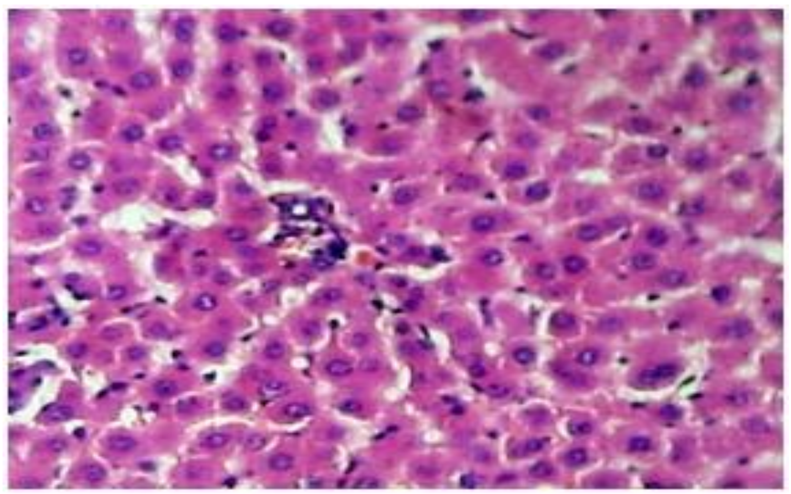
: Normal control
: Toxic control
: Standard
: low dose
High dose

Fig.No.14. Effect of Sylimarin and HASA on LPO, GSH and SOD &CAT in Ethanol induced liver toxicity

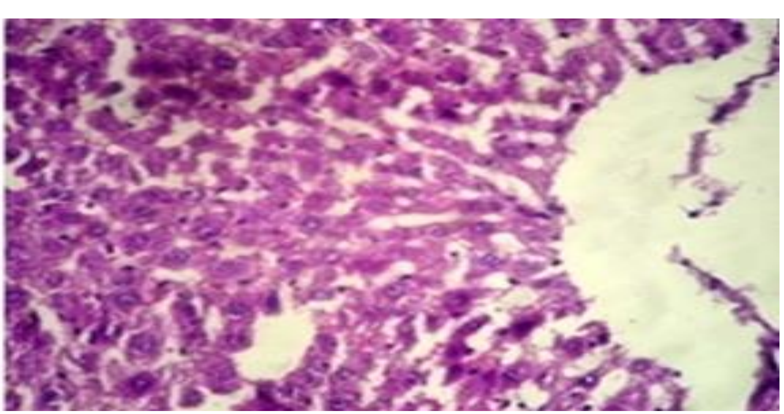
7.4 Histopathological studies of the liver

The histopathological evaluation of Ethanol toxicity in all the groups was examined and shown in Fig.9.Liver section of normal group shows liver parenchyma with intact architecture. Most hepatocytes appear normal. In toxic control group shows inflammation, centrilobular degeneration and necrosis. Treatment with HI (200 & 400 mg/kg) found to reduce inflammation, centrilobular and bridging necrosis. Liver section of this group shows normal hepatocytes with significant reduction in areas of necrosis when compared to toxic group. These changes show protective effect of the drug against hepatic damage induced by Ethanol.

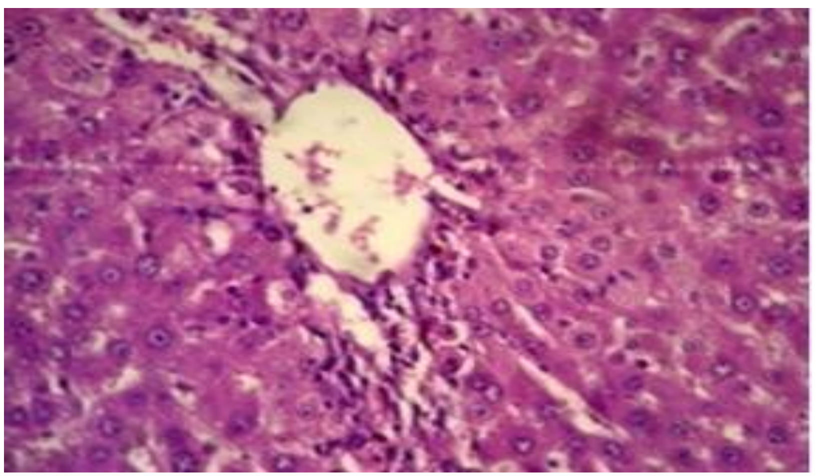
Fig.15: Effect of Silymarin and HI on liver histology in Ethanol induced liver toxicity.



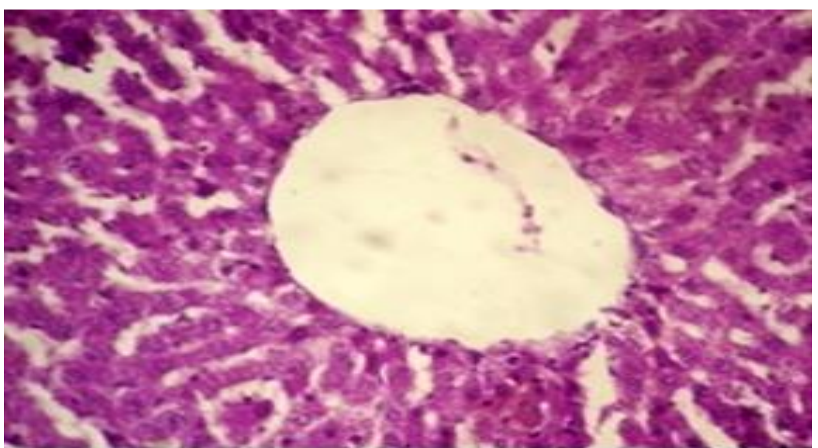
Liver of normal rat.



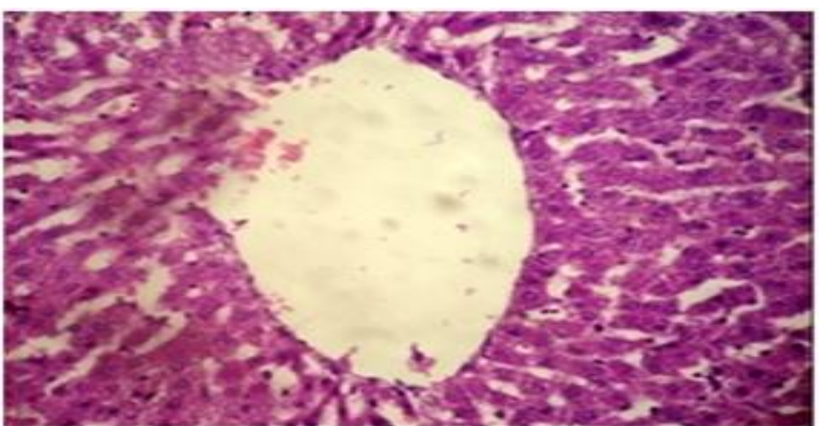
Liver of Ethanol Induced rat.



Liver of silymarin treated rat.



Liver of HI 200mg/kg treated rat.



Liver of HI 400 mg/kg treated rat.

DISCUSSION

8. DISCUSSION

Alcolhol induced hepatic injuries are commonly used models for the screening of hepatoprotective drugs and the extent of hepatic damage is assessed by the level of released cytoplasmic alkaline phosphatase and transaminases in circulation. It is well documented that ethanol is biotransformed under the action of microsomal cytochrome P-450 of liver to reactive metabolites[65].These metabolites attributed to damage structural integrity of liver and raise the levels of SGPT, SGOT, ALP and bilirubin. Further depletion of GSH, decreased protein synthesis, triglycerides accumulation, increased lipid peroxidation results in hepatocyte damage[66]. LD_{50} studies of HI were conducted in albino rats by using OECD guidelines No-425. It was found that extract at 2000 mg/kg dose was safe; hence confirming practically it is safe in nature.

The hydroalcoholic extract prepared was subjected to phytochemical tests and the outcome of these tests revealed the presence of carbohydrate, glycosides, flavonoids, tannins, steroids and saponins.An attempt has been made in the present study to evaluate the hepatoprotective activity of HI against ethanol induced hepatotoxicity in rats. The present study revealed a significant alteration in physical and biochemical parameter after exposure to the Ethanol. The plant extract HI-200 & HI-400 showed dose dependent decrease in the elevated serum biomarkers (SGPT, SGOT, ALP and bilirubin) and endogenous enzymes(GSH, SOD, CAT), it also observed that increase in total protein levels which were comparable to the standard and significant reduction in the level of lipid peroxidation. The number of investigators has reported that flavonoids[67], saponins[68] and other phenolic compounds are known to possess hepatoprotective activity in animals. It is therefore to speculate that the phytoconstituents present in this plant extracts might responsible for the observed hepatoprotective activity.

SUMMARY & CONCLUSION

9. SUMMARY & CONCLUSION

In the present study, the *Hemidesmus indicus* was selected to evaluate its hepatoprotective effect in preclinical models. The *Hemidesmus indicus* was evaluated for hepatoprotective activity in Ethanol induced liver toxicity in experimentally induced hepatotoxic rats. Extraction and preliminary phytochemical studies of *Hemidesmus indicus* revealed the presence of, carbohydrates, steroids, flavonoids, saponins and tannins. Acute oral toxicity study was performed to find out the test dose according to OECD 425 guidelines and HASA was found to be safe at a dose of 2000 mg/kg, b.w.

Hepatoprotective activity was studied against Ethanol induced hepatotoxicity, using silymarin (100 mg/kg) body wt as a standard reference. The physical parameters such as wet liver weight and wet liver volume, biochemical parameters (SGPT, SGOT, ALP, BILD, BILT and TP) levels, endogenous enzymes (GSH, SOD, CAT and LPO) level and histopathology of liver were also studied.

The maximum effect was seen with 400 mg/kg dose of the extract than the 200 mg/kg in hepatoprotective study. The investigation undertaken was aimed to study hepatoprotective activity of *Hemidesmus indicus* demonstrated the usefulness and beneficial effects in the treatment of liver disorder induced by Ethanol. The findings of the present study concluded that *Hemidesmus indicus* witnessed a dose dependent significant hepatoprotective activity.

The treatment with *Hemidesmus indicus* could able to restore the organ (liver) weight and volume at considerable range, which were elevated in hepatotoxic animals, hence proving organ protective activity.The hepatoprotective activity was found to be more significant in high dose (HI-400 mg/kg) compared to low dose (HI-200 mg/kg) in both the animal models. The hepatoprotective potential of *Hemidesmus indicus* in both the experimental model may be due to the presence of flavonoids, saponins and other polyphenolic compounds which are attributed for the antioxidant activity.

The exact mechanism for the hepatoprotective activity of *Hemidsmus indicus* is still unknown and the protection level was found to be at considerable range. Hence further studies are needed to isolate, characterize the active principles and to find out the exact mechanism responsible for its hepatoprotective activity.

BIBLIOGRAPHY

10. BIBLIOGRAPHY

1. Zeashan Hussain, Gupta Amresh, Satyawan Singh, Ch V Rao. Hepatoprotective and antioxidant activity of *Amaranthus spinosus* against CCL_4 induced toxicity. J of ethnopharmacology 125(2):364-6.
2. Wang X, Sun Q, Ding Z. Redefining the molecular organization of the core Mediator complex. Cell Res 24(7):796-808.
3. Kwo P, et al. Simeprevir plus sofosbuvir (12 and 8 weeks) in hepatitis C virus genotype 1-infected patients without cirrhosis: OPTIMIST-1,a phase 3, randomized study. Hepatology.2016;64(2):330-2.
4. Petta S, Muratore C, Craxi A. Non-alcoholic fatty liver disease pathogenesis: the present and the future. Dig Liver Dis. 2009 Sep;41(9):615-25.
5. F Mirzaagha, S H Azali. Coeliac disease in autoimmune liver disease: A Cross-sectional study and a systematic review. Digestive and Liver Disease. 42(9):620-3.
6. Ariff Bongso, Kalamegam Ganthaman. Teratomas from pluripotent stem cells. J. Cell. Biochem. 111:769-781,2010.
7. Vir-Singh Negi, Sriramalu Elluru, Sophie Siberil. Intravenous immunoglobulin clinical use and Mechanisms of Action. J. Clini. Immuno. 233-245.
8. Obot I S, Room R. Alcohol, gender and drinking problems perspective from low and middle income countries. WHO, 2005.
9. Gerd A Kullak-Ublick, Raul J Andrade, Michael Merz. Drug induced liver injury. Gut. 2017; 66:1154-1164.
10. Nancy Vargas Mendoza, Eduargo Madrigal S. Hepatoprotective effect of silymarin. World J. Hepatol. 2014; 6(13):144-149.
11. Suzy M Salama, M H Abdullah, Shahram Golbabapour. Hepatoprotective effect of Ethanolic extract of *Curcuma longa* on thioacetamide induced liver cirrhosis in rats. BMC article no. 56, 2013.
12. Suzy M Salama, M H Abdullah. Mechanism of hepatoprotective effect of *Boesenbergia rotunda* in thioacetamide induced liver damage in rats. Evid. Based. Complement Alternat. Med. 2018; 1072403.
13. M Sengupta, Gauri D Sharma, Biswajit Chakraborty. Hepatoprotective and immunomodulatory properties of aqueous extract of *Curcuma longa* in carbon

tetra chloride intoxicated Swiss albino mice. Asian Pac J Trop Biomed. 2011;1(3): 193-199.

14. Xi Chen. Protective effects of quercetin on liver injury induced by Ethanol. Pharmacogn Mag. 2010; 6(22): 135-141.
15. Warriers P K, Nambiyar VPK & Ramankutty C. *Indian medicinal plants-compendium of 500 species* (Orient Long man, Delhi), Vol 3, 1994,141.
16. Kirtikar KR & Basu BD. *Indian medicinal plants*, 3(1984):1596.
17. Nadkarni KM, Nadkarni AK & Chopra RN. *Indian material medica*. (Popular publication, Bombay), (1954): 619.
18. Satyavathi GV, Gupta AK, Tandon N, *Medicinal plants of India*, Vol 2 (ICMR, New Delhi), 1987.
19. Datta MK, Sen TK, Sikdas S. Some preliminary observations on the anti-inflammatory properties of H. *Indicus* in rats. Indian J Pharmacol, 14(1982):78.
20. Alam MI, Gomes A. Viper venom-induced inflammation and inhibition of free radical formation by pure compound (2-hydroxy-4-methoxy-benzoic acid) isolated and purified from anantamul H. *Indicus* root extract. Toxicon, 36(1998):207.
21. Sivarajan VV, Balachandran I. Ayurvedic drugs and their plant sources (Oxford and IBH publisher, Bombay), 1994.
22. Prabakaran M, Anandan R, Devaki T. Protective effect of H. Indicus against rifampicin and isoniazid induced hepatotoxicity in rats. Fitoterpia, 71(2000):55.
23. Irawan W, Enos T, Yong-ung K. Antimicrobial and Antioxidant Properties of Medicinalplants Used by the Bentian Tribe from Indonesia. Food science and human wellness. 2015; 5(2): 24-67.
24. Rajasekaran C, Kalaivani T, Ramya S, Jayakumararaj R. Studies on Hepatoprotective Activity of Ethanolic Extracts of Fruit pulp of Aegle marmelos (L.)Corr. Journal of Pharmacy Research. 2009; 2(7): 1419-1423.
25. Sanjay KJ, Sourabh R, Prashant D, G.K. Singh, Nagori BP. Herbal Extract as Hepatoprotective-A Review. Journal of Pharmacognosy and Phytochemistry 2013; 2 (3): 170-175.
26. Racenelli V, Rehermann B, The liver is an immunological organ. J Hepato, 2016; Vol 43:S54- S62.

27. Bioulac Sage P, Rebouissou S, Thomas C. Hepatocellular adenoma sub type classification using molecular markers and immunohistochemistry. Hepatology, 2007; 46(3):740-8.
28. Andrew W Duncan, Craig Dorrell, Markus Grompe. Stem cells and Liver generation. 2009; Vol 137, 2: 466-481.
29. Taub R. Liver regeneration from myth to mechanism. Nat Rev Mol Cell Biol. 2004; 5:836–847.
30. Wang Y, Li J, Wang X. Hepatic stellate cells, liver innate immunity and hepatitis C virus. J Gastroentrol Hepatol, 2013; 1: 112-5.
31. Ian Nicholas Crispe. The liver as a lymphoid organ. Annual review of Immunology. Vol 27; 2009:147-163.
32. Sanjay Chanda, Harihara M Mehendale. Hepatic cell division and tissue repair. Molecular medicine today. Vol 2; 1996: 82-89.
33. Keppler D, Lesch NR, Reutter W, Decker K. Experimental hepatitis induced by Dgalactosamine. Exp Mol Path. 1968; 9: 279- 290.
34. Mirelle S Farias, Patricia Budni, Carine M Ribeiro. Antioxidant supplementation attenuates oxidative stress in chronic hepatitis C patients. J Gastroenterol Hepatol. Vol 3; 2012: 386-394.
35. Abdul-Hussain, S.K., Mehendale, H.M. Studies on the age dependant effect ofgalactosamine in primary rat hepatocyte cultures. *Toxicology and Applied Pharmacology*.1991; 107; 504–513.
36. Beers RF, Sizer IW. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase1952; 195:133-40.
37. Bhandarkar,M.R., Khan, A. Antihepatotoxic effect of *Nymphaea stellata* willd., againstcarbon tetrachloride-induced hepatic damage in albino rats. *Journal of Ethnopharmacology*. 2004; 91: 61–64.
38. Bhandarkar,M.R., Khan, A. Antihepatotoxic effect of *Nymphaea stellata* willd., againstcarbon tetrachloride-induced hepatic damage in albino rats. *Journal Ethnopharmacology*. 2004; 91; 61–64.
39. Cartwright, C.K., Ragland, J.B., Weidman, S.W., Sabesin, S.M., Alterations in Lipoprotein composition associated with galactos amine induced rat liver injury.*Journalof Lipid Research.*1982; 23; 667–679.

40. Chattopadhyay R.R. Possible mechanism of hepatoprotective activity of *Azadirachtaindica* leaf extract: Part II. *Journal of Ethnopharmacology*. 2003; 89; 217–219.
41. Djehuty, M. R. (2007). *Candida albicans*: Yeast infection. Comp. of Derm. Herbs, 17:114-120.
42. Tortora GJ, Bryan Derrickson. Principles of anatomy and physiology, 12th ed New York. John Willy and Sons NC. P.945-9.
43. Fauci SA, Braunwald E, Dennis L Kasper, Stephen LH, Longo DL, Jameson JL et al . Principles of Harrison's internal medicine. 7th Ed. 1918.
44. Thomas C, Pellicciari R, Pruzanski M, Auwerx J, Schoonjans K. Targeting bileacid signalling for metabolic diseases. Nat Rev Drug Discov 2008; 7:p.678–93.
45. Sharma A., Chakraborti K.K., Handa S.S. Anti-hepatotoxic activity of some Indian herbal formulations as compared to silymarin. Fitoterapia. 1991; 62: 229-235.
46. Subramonium A., Pushpangadan P. Development of Phytomedicines for liver diseases. Indian J Pharmacology. 1999; 31: 166- 175.
47. Ostapowicz G., Fontana R.J., Schiodt F.V., Larson A., Davron J.T., Steven H.B., Timothy M., Reish J. Results of a prospective study of acute liver failure at 17 tertiary care centers in the United States. Ann Intern Med. 2002; 137: 947–954.
48. Warriers P K, Nambiyar VPK & Ramankutty C. *Indian medicinal plants-compendium of 500 species* (Orient Long man, Delhi), Vol 3, 1994,141.
49. Verma, Prashant R.; Joharapurkar, Amit A.; Chatpalliwar, Vivekanand A.; Asnani, Alpana J. (November 2005). Antinociceptive activity of alcoholic extract of Hemidesmus indicus R.Br. in mice. *Journal of Ethnopharmacology*. **102** (2): 298–301.
50. Lakshman K, Jaiprakash B, Shivaprasad HN, Anti-inflammatory and Antipyretic activities of Hemidesmus indicus root extract. African J Tradi. Vol 3, 10:4314.
51. Khan BA, Akhtar N, Rasul A, Tariq M, Khan MS, Shahiq Z et al. Investigation of the effects of extraction solvent/technique on the antioxidant activity of Cassia fistula L. J Med Plants Res 2012;6(3):500-3.

52. Trease GE, Evans MC. Text book of Pharmacognosy. London, Bailliare Tindall. 1983; 12:p193-336.
53. Kokate C K. Practical Pharmacognosy. 1ed New Delhi, Vallabh Prakashan. 1994; 4:p110-11.
54. OECD/OCDE. 425 OECD guidelines for testing of chemicals acute oral toxicity, up and down procedure 2001; 26:1-26.
55. German society for clinical chemistry, Z. Klin. Chem. Kin. Biochem. 1972; 10: 281.
56. IFCC methods for the measurement of catalytic concentration of enzymes. J Clin Chem Clin Biochem 1986;24:481-97.
57. Bowers GN, Mc Commb RB. Recommendations of the German society for clinical chemistry. J Clin Chem Bi 1972; 10:182.
58. C J Hsueh, Joanne H. Wang, Liming Da, CC Liu. Determination of Alanine Aminotransferase with an Electrochemical Nano Ir-C Biosensor for the Screening of Liver Diseases. Biosensors 2011;1: 107-17.
59. Pearlman PC, Lee RT. Detection and measurement of total bilirubin in serum with use of surfactants as solubilising agents. Clin Chem 1974;20: 447.
60. Gornall A.Determination of serum proteins by means of the biuret reaction. J Biol Chem 1949; 177:751.
61. Balaraman R, Bafna PA, Kolhapure SA. Antioxidant activity of DHC-1* – a herbal formulation. J Ethnopharmacol 2004; 94:135-41.
62. Oyaizu M,. Studies on product of browing reactions preparations from glucoseamine. Jap J Nutrition 1986; 44:307-9.
63. S. O. Oyedemi, G. Bradley and A. J. Afolaya .In -vitro and In-vivo antioxidant activities of aqueous extract of *Strychnos henningsii Gilg*. African J Pharmand & Pharmacol 2010; 4(2):70-8.
64. Karunakar Hegde, Arun B Joshi. Hepatoprotective effect of *Carissa carandas* Linn root extract against CCl_4 and paracetamol induced hepatic oxidative stress. Indian J Expt Biol 2009; 47:660-7.
65. Syed Aamir, Thippeswamy B S, Kulkarni V H, Hegde Karunakar. Hepatoprotective effect of *Euphorbia thymifolia* whole plantextract on CCl_4 induced hepatic damage in rats. Int J Res Ayurveda Pharm 2011;2(2):681-6.

66. Savides M C & Woehme F.Acetaminophen and its toxicity.J Appl Toxicol 1983; 3:96.
67. Paya M, Ferrandiz M L, Sanz M J & Alcaraz M J. Effects of phenolic compounds on bromobenzene mediated hepatotoxicity in mice. Xenobiotica1993; 23:327.
68. Tran Q I, Adnyana I K, Tezuka Y, Nagaoka T, Tran Q K & Kadota S.Triterpene saponins from *Vietnamese ginseng*(Panaxvietnamensis) and their hepatocyte protective activity. J Nat Prod 2001; 64: 456.