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AUTHENTIFICATION CERTIFICATE

I hereby certify that the following plant species for pharmacology / Pharmacognostic studies/Chemical investigation research work, brought by him/her is identified and their botanical name and family name is given. The Plant species is not harmful and for research purpose only.

Botanical name	Family
Wedelia Calendulacea	*Asteraceae*

Authenticated by

K. Madhava Chetty

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Dedicated
To
My parents and guide

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At the end of our thesis, we would like to thank all those people who made this thesis possible and an unforgettable experience for us. At the end of our thesis, it is a pleasant task to express our thanks to all those who contributed in many ways to the success of this study and made it an unforgettable experience for us.

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CONTENTS

LIST OF FIGURES

LIST OF TABLES


INTRODUCTION

1. INTRODUCTION

Liver is the heaviest gland of the body weighing about 1.4 kg in an average adult and is inferior to the diaphragm occupying most of the right hypochondriac and a part of the epigastric region of abdominopelvic cavity. It is the largest and most complex organ in the human body and has a unique and remarkable power to regenerate. It carries out a wide range of exocrine and endocrine functions such as maintenance of carbohydrate homeostasis, storage of nutrients, secretory and excretory functions, protein synthesis and a couple of vital metabolic processes including hormone, lipid and protein metabolism and detoxification of xenobiotics mediated by cytochrome P450-dependent oxidases and conjugates (Guyton *et al*., 1981). The sodium and potassium salts of bile acids play an important role in emulsification and breakdown of large lipid globules into a suspension of droplets and also in the absorption of lipids following their digestion (Kumar P *et al.,* 2008). The wide range of its functions makes the liver vulnerable to a variety of disorders which can be broadly classified into acute or chronic hepatitis (inflammatory disease), hepatosis (non-inflammatory disease) and liver cirrhosis (Wagner *et al*., 1980).

Thus, liver diseases are some of the fatal diseases in the world today. They pose a serious challenge to international public health. The drugs available in the modern system of medicine are corticosteroids and which bring about symptomatic relief without cure and their use is associated with the risk of relapses and dangerous side effects (Guyton *et al*., 1981).

The liver takes part in the metabolism, detoxification and secretion functions in the body. It is the major target organ of chemical-induced toxicity. Liver damage in most cases involves oxidative stress and is characterized by progressive evolution from steatosis to chronic hepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma. It has been found that the metabolism of carbon tetrachloride (CCl4) involves production of the highly lethal trichloro methyl radical (•CCl3) and peroxy trichloro methyl (•OOCCl3) free radical through activation by drug metabolizing enzymes located in the endoplasmic reticulum. CCl4 can cause lipid peroxidation as well as deposition of the extracellular matrix (ECM), resulting in liver cirrhosis.

Clinical and experimental examinations have shown that cirrhosis can be reversed. Various pharmaceutical drugs have been used to minimize and reverse the insult, but most of them lead to appreciable side-effects during long-term treatment. In this context, the use of an effective alternative without side-effects is crucial to reduce the oxidative stress which leads to hepatic disorders. Currently, there is great awareness of the health benefits of phenolic and polyphenolic compounds because of their antioxidant potential. Dietary plants possessing phenolic and polyphenolic compounds have been shown to exert various biological actions. These include the scavenging of free radicals, metal chelation, increases in enzymatic activity. More recently, they have been shown to influence signal transduction, release of transcription factors, and gene expression.

Several reasons are known to cause moderate to severe hepatic complications. Few liver complications emerge out as result of socially unaccepted life style of the individuals. On the other hand, some other liver toxicities result due to unavoidable circumstances. Chronic alcoholism is one of the very commonly known avoidable causes of liver damage such as fatty infiltration, hepatomegaly and cirrhosis. On the other hand, environmental pollution and exposure of the individual to various hepatotoxic substances due to occupational responsibilities also produce hepatotoxicity.

Further, compounds including clinically useful drugs can cause cellular damage through the metabolic activation of the parent compound to highly reactive substances and also provoking the generation of oxygen derived free radicals. Nimesulide (4-nitro-2-phenoxymethane-sulfoanilide) is such a non-carboxylic acid, non-steroidal anti-inflammatory drug that has been widely used for the treatment of a variety of inflammatory and pain conditions. If the drug is consumed in overdoses or for longer period of time, people having weak liver function suffer severely from unpredictable hepatic problems. It has been reported that the drug can cause several types of liver damage, ranging from mild abnormal function such as increase in serum amino transferase activity to severe organ injuries such as hepatocellular necrosis or intrahepatic cholestasis1. Indeed, drug induced liver toxicity is the leading cause of acute liver failure in the United States, according to the Food and Drug Administration (FDA).

According to WHO about 18,000 people die every year due to liver diseases. The common ailments of liver are cirrhosis, cholestasis, hepatitis, portal hypertension, hepatic encephalopathy, fulminant hepatic failure and certain tumors like hepatoma. It is estimated that two billion people around the world are infected with hepatitis B. About 350 million of these have the chronic form of the disease. This alarming statistics with perplexing report, warrant the immediate necessity of studies of any level to either ensure the effectiveness of available formulations or exploration of the new herbal therapies to reduce the morbidity and mortality rate due to hepatic complications (Jain A *et al.,* 2008).

In modern medicine corticosteroids and immune suppressants are commonly used to treat liver disease in allopathic form of medicine. But these drugs are associated with adverse effects such as immunosuppression and bone marrow depression. Further, the success rate of treating liver diseases is disappointing. Attempts are being made globally to get scientific evidences for this traditionally reported herbal drug (Anurekha *et al.,* 2007).

In view of severe undesirable side effects of synthetic agents and absence of reliable liver protective drugs in the modern medicine, there is growing focus to evaluate scientific basis for the use of traditional herbal medicines which are claimed to possess hepatoprotective activity. About 70-80% of the world populations rely on the use of traditional medicine, which is predominantly based on plant materials. The traditional medicine refers to a broad range of natural health care practices including Ayurveda, Siddha, Homeopathy and Unani. Bile is partially excretory product and partially a digestive secretion from hepatocytes. (Harsh Mohan *et al.,* 2007**).**

Traditional healers and pharmacist in developing countries are in information about plant sources of new drugs only a fraction of the earth natural pharmacopoeia has been analyzed with modern techniques ethano pharmacology can also be an important element of a developing nation's medical and economic system. Third world government is being encouraged to seek synthesis between modern and traditional Medicine.

In this direction to support the ayurvedic recommendation, we designed the present research with the objective of hepatoprotective activity of *wedelia calendulacea* leaves against carbon tetrachloride induced chronic hepatitis.

Wedelia calendulacea is a shrub or small tree belonging to the family Asteraceae. It was found in all over India. The plants are being used to treat liver disorders, uterine hemorrhage and menorrhagia (A.K. Meena *et al*., 2011). The plant also shows analgesic and anti-inflammatory activity (Bhargava *et al*., 1982). It is also used to treat skin problems, dermatitis, eczema and acne (Hegde DA *et al*., 1994). Recently it is reported that it possesses antimicrobial activity, antifungal activity. Leaves in decoction are antiscabious, Decoction of roots and leaves for stomach aches and fevers. Leaves used for cleaning and dressing ulcers, Juice of leaves, with cow's milk, taken as tonic after childbirth. Decoction of leaves with ginger for flatulence, Flowers are known to be purgative. A study of the n-hexane extract of *Wedelia calendulacea* showed antibacterial activity against Bacillus subtilis, Mycobacterium smegmatis, Pseudomonas aeruginosa, Salmonella group C, S Para typhi and Shigella sonnei (Taddei A *et al*., 1999).

Liver is the major site of xenobiotic metabolism in any vertebrate living system. Therefore, injury to liver caused by toxic chemicals, drugs and virus infiltration from ingestion or infection may be harmful and can lead to various complications (Ghosh *et al.,* 1996). Hepatic disorders have far reached consequences, given the critical dependence of other organs on the metabolic functions of the liver. Liver injury and its manifestations tend to follow characteristic patterns. In some instances, the diseased process is primary to the liver.

1.1 ANATOMY OF THE LIVER:

The liver is almost completely covered by visceral peritoneum and is completely covered by dense irregular connective tissue layer that lies deep to the peritoneum. The liver is divided into two principal lobes – a large right lobe and a smaller left lobe – by the falciform ligament. Even though the right lobe is considered by many anatomists to include an inferior quadrate lobe and a posterior caudate lobe, on the basis of internal morphology, the quadrate and caudate lobes more appropriately belong to the left lobe. The falciform ligament, a fold of the parietal peritoneum, extends from the undersurface of the diaphragm between the two principal lobes of the liver to the superior surface of the liver, helping to suspend the liver. In the free border of the falciform ligament is the ligamentum teres (round ligament), a fibrous cord that is a remnant of the umbilical vein of the fetus, which

extends from the liver to the umbilicus. The right and left coronary ligaments are narrow reflections of the parietal peritoneum that suspend the liver from the diaphragm. (Guyton *et al.*, 2000)

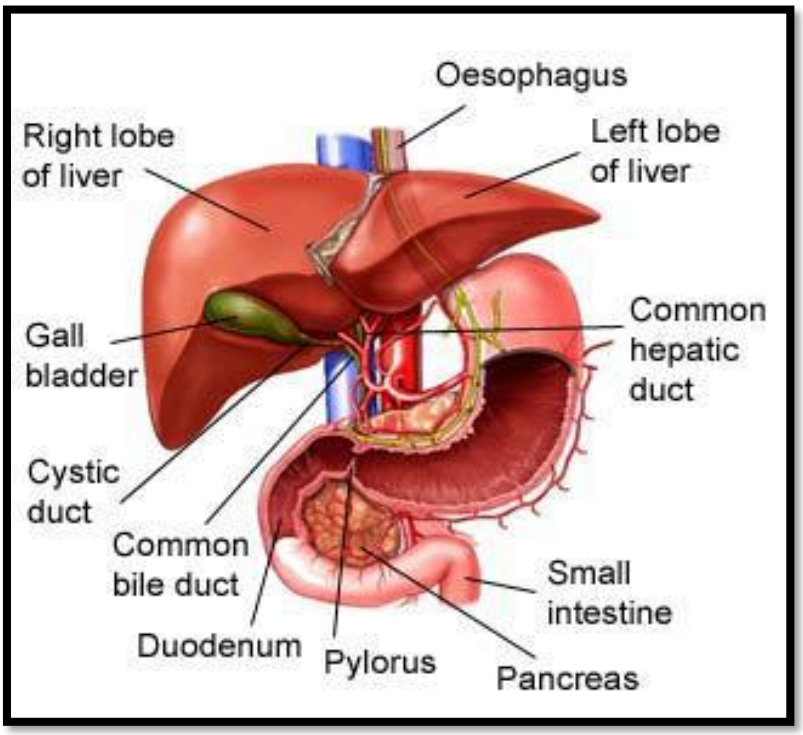


Fig 1: Liver

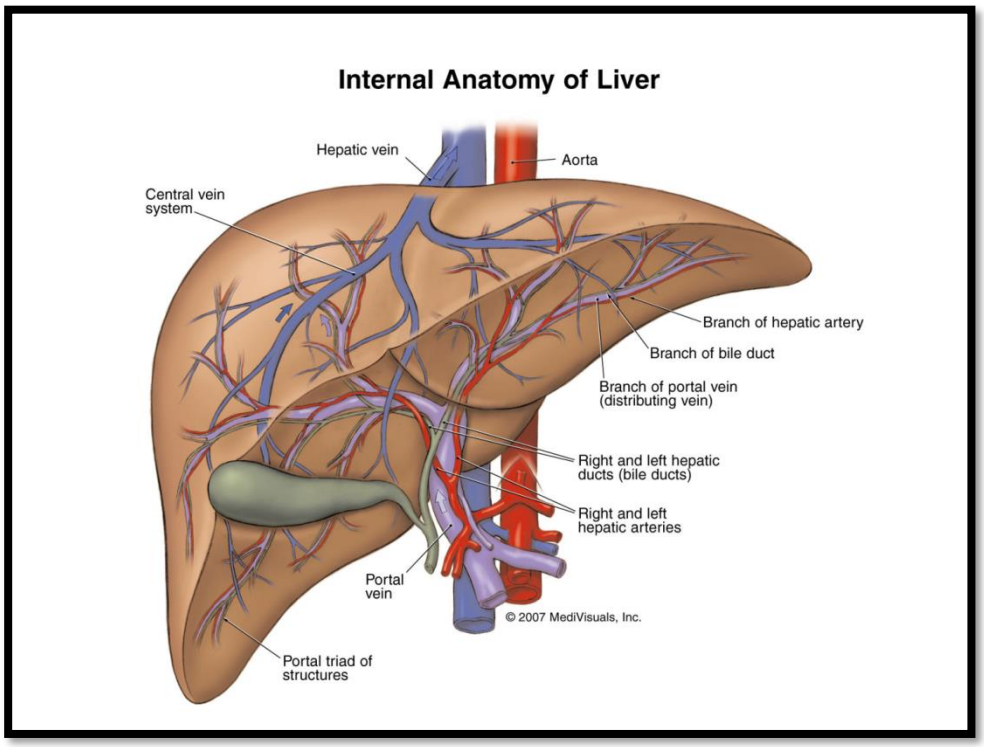


Fig 2: liver internal anatomy

1.2 HISTOLOGY OF THE LIVER:

The lobes of the liver are made up of many functional units called lobules. A lobule consists of specialized epithelial cells, called hepatocytes (hept=liver, cytes=cells), arranged in irregular, branching, interconnected plates around a central vein. Instead of capillaries the liver has large, endothelium lined spaces called sinusoids, through which blood passes. Also present in the sinusoids are fixed phagocytes called stellate reticuloendothelial (Kupffer's) cells, which destroy worn out leukocytes and red blood cells, bacteria, and other foreign matter in the venous blood draining from the gastrointestinal tract.

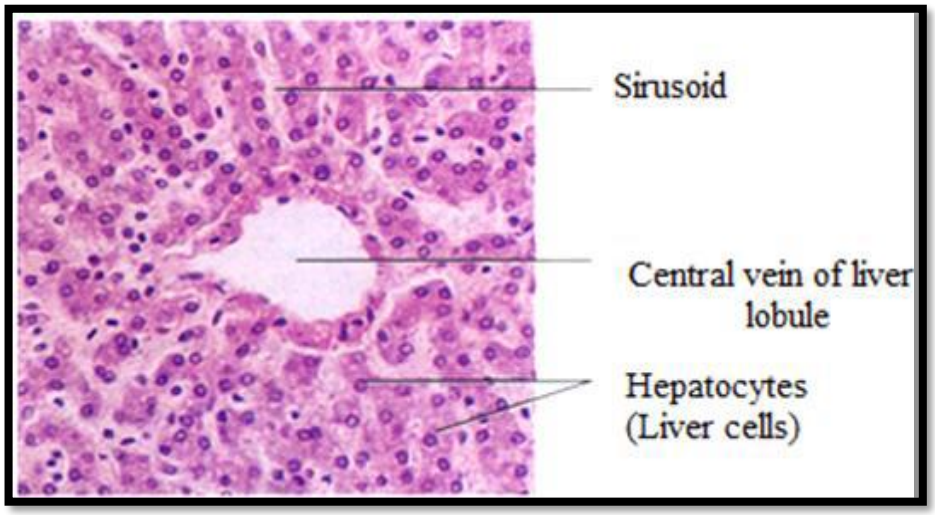


Fig 3: Portion of a liver lobule

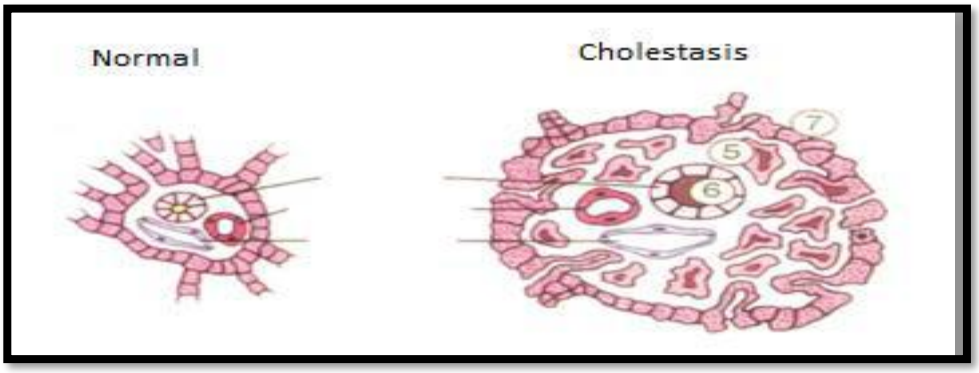


Fig 4: Parenchyma

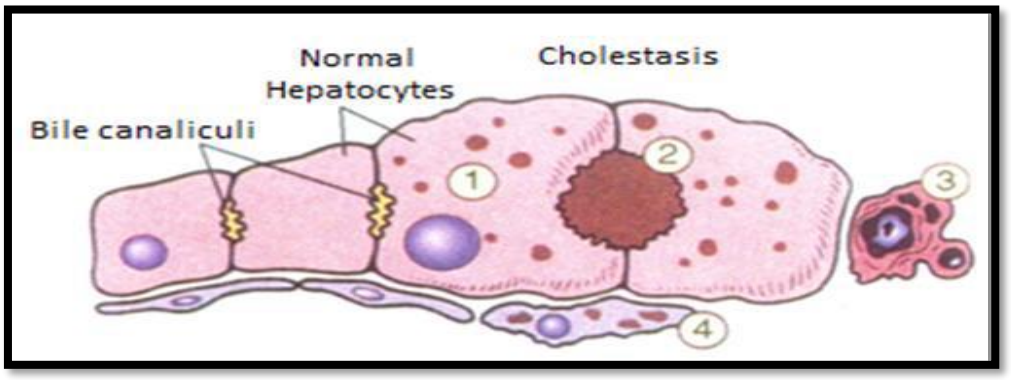


Fig 5: Portal tract

1.3 BLOOD SUPPLY TO THE LIVER:

The liver receives blood from two sources. From the hepatic artery it obtains oxygenated blood, and from the hepatic portal vein it receives deoxygenated blood containing newly absorbed nutrients, drugs, and possibly microbes and toxins from the gastrointestinal tract. Total human liver blood flow represents approximately 25% of the cardiac output up to 1500 ml/min. Hepatic flow is subdivided in 25-30% for the hepatic artery (500ml/min) and the major part for the portal vein (1000ml/min). A second blood supply to the liver comes from the hepatic artery, branching directly from the celiac trunk and descending aorta (Shalam *et al.*, 2007).

The portal vein supplies venous blood under low pressure conditions to the liver, while the hepatic artery supplies high-pressured arterial blood. Since the capillary bed of the gastrointestinal tract already extracts most O2, portal venous blood has a low O2 content. Blood from the hepatic artery on the other hand, originates directly from the aorta and is, therefore, saturated with O2. Blood from both vessels joins in the capillary bed of the liver and leaves via central veins to the inferior canal vein.

1.4 FUNCTIONS OF THE LIVER:

Besides secreting bile, which is needed for absorption of dietary fats, the liver performs many other vital functions:

1. Carbohydrate metabolism: The liver is especially important in maintaining a normal blood glucose level. When blood glucose level is low, the liver can break down glycogen to glucose and release glucose into the bloodstream. The liver can also

convert amino acids into lactic acid and galactose into glucose when blood glucose is high, as occurs just after eating a meal, the liver convert's glucose to glycogen and triglycerides for storage.

2. Lipid metabolism: Hepatocytes store some triglycerides, break down fatty acids to generate ATP, synthesize lipoproteins, which transport fatty acids, triglycerides and cholesterol to and from body cells, synthesize cholesterol and use cholesterol to make bile salts.

3. Protein metabolism: Hepatocytes deaminate (remove the amino group, NH2 from) amino acids so that the amino acids can be used for ATP production or converted to carbohydrate or fats. The resulting toxic ammonium is then converted into the much less toxic urea, which is excreted in urine. Hepatocytes also synthesize most plasma proteins, such as alpha and beta globulins, albumin, prothrombin and fibrinogen.

4. Detoxification: Detoxification Drug metabolism is an important liver function. It is a complex process that occurs in the endoplasmic reticulum of the hepatocyte. Several phases are involved with this detoxification:

a) **Phase - I reaction:** In the phase 1 reaction, oxidation or demethylation occurs, mediated by cytochrome P450. A variety of oxidative phase 1 reactions are performed by the enzymes that make up the P450 system. Found primarily in the liver but also in the gastrointestinal tract, kidneys, brain and other tissues. P450 enzymes are composed of a unique apoprotein and a heme prosthetic group, which binds oxygen after electron transfer reactions from NADPH, resulting in aliphatic and aromatic hydroxylation, O, N, or S dealkylation or dehalogenation. A typical reaction of this type generates a hydroxyl group, which can then participate in the phase 2 reactions. Each group of genes with 40 percent amino acid homology composes a family whose gene products (isozymes) may function in a similar fashion. For example, CYP3 is a family that contains an A subfamily and several genes, numbered 1, 2, and so forth. The primary enzyme for the metabolism of erythromycin in humans is P450 3A4.

b) **Phase II reaction:** After a phase 1 reaction, most compounds are still not very water-soluble and require further metabolism. In a typical phase 2 reaction, a large water-soluble polar group is attached to hydroxyl oxygen by glucuronidation or sulfation, forming ether or ester linkages. These are the sole steps required for the hepatic metabolism of some compounds. But for

most, the phase 2 reaction is preceded or followed by phase 1 and furosemide, as well as bilirubin. Sulfation is as important as glucuronidation particularly for the metabolism of steroid compounds and bile acids. There are several species of sulfotransferases with overlapping specificities, each employing 3phosphoadenosine-5-phosphosulfate synthesized from ATP and sulfate ions. Although, phase 2 reactions are usually accomplished without a detrimental effect, they can occasionally lead to toxic or carcinogenic by products (Zimmarmann *et al.*, 1976).

5. Excretion of bilirubin: Bilirubin, derived from the blood cells is absorbed by the liver from the blood and secreted into bile. Most of the bilirubin in bile is metabolized in the small intestine by bacteria and eliminated in faeces.

6. Synthesis of bile salts: Bile salts are used in the small intestine for the emulsification and absorption of lipids, cholesterol, phospholipids and lipoproteins.

7. Storage: in addition to glycogen, the liver is a prime storage site for certain vitamins (A, B12, D, E, and K) and minerals (iron and copper), which are released from the liver when needed elsewhere in the body.

8. Phagocytosis: The stellate reticuloendothelial (Kupffer's) cells of the liver phagocytose aged red blood cells and white blood cells and some bacteria.

9. Red Blood Cell System: The liver removes old or damaged red blood cells from the circulation and is involved with the storage of iron and the breakdown of hemoglobin. Because of this, chronic liver disease could cause anemia. The liver (along with the spleen) is a storage organ for blood. If these are a severe blood loss the liver expels this blood into the bloodstream to help make up for the loss.

10. Reticulo endothelial System: Specific cells called Kupffer cells line the inside of the liver. These cells are part of the immune system those, eliminate and degrade the substances that are brought to the liver by the portal vein. A diseased liver will not filter these compounds normally, resulting in toxic accumulations of drugs, chemicals, or bacteria. Excess accumulation of bacteria in the bloodstream causes septicemia liver.

Excess accumulation of bacteria in the bloodstream is called septicemia and is one of the reasons that antibiotics are commonly used in liver disease.

11. Vitamins: Many vitamins are stored in the liver, and perform their functions only when activated by the liver, and are also degraded by the liver. These include some of the B complex vitamins and vitamin C, along with A, D, E.

1.5 PATHOPHYSIOLOGY OF LIVER:

Icterus (jaundice) is the most common disease of liver. It presents as yellow discoloration of the sclera of the eyes, skin and mucus membrane due to build up of bilirubin in the body. Jaundice can have a pre-hepatic, hepatic, or post-hepatic cause:

a) **Pre hepatic:** Diseases that cause extensive red blood cell destruction i.e., immune mediated hemolytic anemia, can overload the liver's ability to metabolize bilirubin. The liver is not diseased in this situation, but it is just being overloaded with work to do. It is a problem because the anemia that causes this overload is a sign of a problem somewhere in the body. It usually takes a severe anemia to cause this problem

b) **Hepatic:** Icterus can also be caused by impaired excretion of bilirubin in a diseased liver. The swelling i.e., cholangiohepatitis, impairs the liver's ability to excrete bilirubin in to the digestive system. The bilirubin builds up and eventually spills over into the causing the discoloration

c) **Post hepatic:** Obstruction of bilirubin flow out of the liver, which is a more extreme version of impaired excretion above, can also cause Icterus. An obstruction of the gall bladder or common bile duct can cause this. These animals will have light colored feces because no bile pigment is being excreted into the digestive system to give stool its dark color. Bilirubin that is retained in the liver is toxic and will add to the liver problem already present (Harsh Mohan *et al.*,2000).

1.6 HEPATIC INJURIES:

In hepatic injury five general responses are seen, viz;

1) Inflammation: Injury to hepatocytes associated with an influx of acute or chronic inflammatory cells into the liver is termed hepatitis. Attack of viable anti gene expressing liver cells by sensitized T-cells is a common cause of liver damage. Inflammation may be limited to portal tract or may spill over into the parenchyma (Ramachandra *et al.*, 2007).

E.g.: viral hepatitis due to hepatitis A virus (HAV), HBV, HCV, HDV and HEV

2) Degeneration: The hepatocytes get damaged due to toxic or immunological insult and show an edematous appearance. Degeneration can also be in the form of steatosis, where in there is accumulation of fat droplets within the hepatocytes.

E.g.: hepatic degeneration can be due to genetic diseases or exogenous substance such as alcohol.

3) Cell death: Cell death which is toxic or immunologically mediated occurs via apoptosis wherein the hepatocytes become shrunken, pyknotic, and intensely eosinophilic. Alternatively, hepatocytes may also undergo lytic necrosis (osmotically swell and rupture). The other types are centrilobular necrosis, bridging necrosis, sub massive necrosis and massive necrosis.

4) Fibrosis: Fibrotic tissue is formed in response to inflammation or direct toxic insult to the liver. Deposition of collagen has lasting consequences on hepatic pattern of blood flow and perfusion of hepatocytes. Initially fibrosis may develop within or around portal tracts or the central vein or may be deposited directly with in the sinusoids. Progressively, these fibrous strands link regions of the liver (portal-to-portal, portal-to-central, central-to-central), a process called bridging fibrosis. Fibrosis is generally considered as an irreversible consequence of hepatic damage.

5) Cirrhosis: Cirrhosis with continuing fibrosis and parenchymal injury, the liver is subdivided into nodules of degenerating hepatocytes surrounded by scar tissue, termed cirrhosis and is an end stage form of liver.

The clinical consequences of liver diseases are hepatic dysfunction in the form of jaundice, hypoalbuminemia, hyperammonemia, hyperglycemia, fetor hepatitis, palmar erythema, spider angiomas, hypogonadism, gynecomastia, weight loss, muscle wasting, and portal hypertension from cirrhosis. If these are not treated promptly, they will lead to life threatening complications like hepatic failure in the form of hepatic encephalopathy, hepatorenal-syndrome; or portal hypertension from cirrhosis, malignancy with chronic disease and hepatocellular carcinoma (Kagan *et al.,* 1998).

Liver damage is always associated with cellular necrosis, increase in tissue lipid peroxidation and depletion in the tissue GSH levels. In addition, serum levels of many biochemical markers like SGOT, SGPT, triglycerides, cholesterol, bilirubin, alkaline phosphatase are elevated.

1.7 LIVER FUNCTION TESTS

Liver has to perform different kinds of biochemical, synthetic and excretory functions, so no single biochemical test can detect the global functions of liver. All

laboratories usually employ a battery of tests for initial detection and management of liver diseases and these tests are frequently termed “Liver function tests”, although they are of little value in assessing the liver.

The clinical history and physical examination play important role to interpret the functions. Moreover, the role of specific disease markers, radiological imaging and liver biopsy cannot be underestimated (Daniel *et al.*, 1999).

The various uses of Liver function tests include:

1. **Screening:** They are a non-invasive yet sensitive screening modality for liver dysfunction.
2. **Pattern of disease:** They are helpful to recognize the pattern of liver disease. Like being helpful in differentiating between acute viral hepatitis and various cholestatic disorders and chronic liver disease. (CLD).
3. **Assess severity:** They are helpful to assess the severity and predict the outcome of certain diseases like primary biliary cirrhosis.
4. **Follow up:** They are helpful in the follow up of certain liver diseases and also helpful in evaluating response to therapy like autoimmune hepatitis.

Limitations:

1. **Lack sensitivity:** The LFT may be normal in certain liver diseases like cirrhosis, non-cirrhotic portal fibrosis, congenital hepatic fibrosis, *etc.*
2. **Lack specificity:** They lack specificity and are not specific for any particular disease. Serum albumin may be decreased in chronic disease and also in nephritic syndrome. Aminotransferases may be raised in cardiac diseases and hepatic diseases. Except for serum bile acids the LFT are not specific for liver diseases and all the parameters may be elevated for pathological processes outside the liver.1,3 Thus, we see that LFT have certain advantages as well as limitations at the same time. Thus, it is important to view them keeping the clinical profile of the patient in mind.

CLASSIFICATION OF LIVER FUNCTION TESTS

1. SERUM BILIRUBIN: Bilirubin is an endogenous anion derived from hemoglobin degradation from the RBC. The classification of bilirubin into direct and indirect bilirubin is based on the original van der Bergh method of measuring bilirubin. Bilirubin is altered by exposure to light so serum and plasma samples must be kept in dark before measurements are made. When the liver function tests are abnormal and

the serum bilirubin levels more than 17μmol/L suggest underlying liver disease (Friedman *et.al.,* 2003).

i. Total bilirubin: This is measured as the amount, which reacts in 30 minutes after addition of alcohol. Normal range is 0.2-0.9 mg/dl (2-15μmol/L). It is slightly higher by 3-4 μ mol/L in males as compared to females. It is this factor, which helps to diagnose Gilbert syndrome in males easily.

ii. Direct Bilirubin: This is the water-soluble fraction. This is measured by the reaction with diazotized sulfanilic acid in 1 minute and this gives estimation of conjugated bilirubin. Normal range 0.3mg/dl (5.1μmol/ L).

iii. Indirect bilirubin: This fraction is calculated by the difference of the total and direct bilirubin and is a measure of unconjugated fraction of bilirubin.1,5 The diazo method of bilirubin estimation is not very accurate especially in detecting low levels of bilirubin. Direct bilirubin over estimates bilirubin esters at low bilirubin levels and under estimates them at high concentration. Thus, slight elevation of unconjugated bilirubin not detected, which is of value in detecting conditions like Gilbert syndrome.5 A newer highly accurate method of estimation involves alkaline methanolysis of bilirubin followed by chloroform extraction of bilirubin methyl esters and later separation of these esters by chromatography and spectrophotometric determination at 430 nm (Rosalki *et al.,* 1999).

Diagnostic value of bilirubin levels:

Bilirubin in body is a careful balance between production and removal of the pigment in body. Hyperbilirubinemia seen in acute viral hepatitis is directly proportional to the degree of histological injury of hepatocytes and the longer course of the disease.

Hyper bilirubinemia:

It results from over production impaired uptake, conjugation or excretion / regurgitation of unconjugated or conjugated bilirubin from hepatocytes to bile duct.

2. URINE BILIRUBIN

The presence of urine bilirubin indicates hepatobiliary disease. Unconjugated bilirubin is tightly bound to albumin and not filtered by the glomerulus and thus not present in urine. Measurable amounts of conjugated bilirubin in serum are found only in hepatobiliary disease.1 because the renal threshold for conjugated bilirubin is low and the laboratory methods can detect low levels of bilirubin in urine so conjugated

bilirubin may be found in urine when the serum bilirubin levels are normal. This is the case in early acute viral hepatitis.1, 6 Tests strips impregnated with diazo reagent are easy to use and detect as little as 1-2m mol bilirubin/L (Rosalki *et al.,* 1999).

Urobilinogen

An increase in the urobilinogen in urine is a sensitive indicator of hepatocellular dysfunction. It is a good indication of alcoholic liver damage, well compensated cirrhosis or malignant disease of the liver. In viral hepatitis it appears early in urine. It is markedly increased in hemolysis. (Rosalki *et al.,* 1999).

In cholestatic jaundice urobilinogen disappears from urine. It may be intermittently present in case of gallstones (Sherlock *et al*., 1997).

Urobilinogen gives a purple reaction to Ehrlich's aldehyde reagent. A dipstick containing this reagent allows rough and ready quantification. Freshly voided urine should be used.

1.8 ENZYMES THAT DETECT HEPATOCELLULAR NECROSIS – AMINOTRANSFERASES

The amino transferases (formerly trans aminases) are the most frequently utilized and specific indicators of hepatocellular necrosis. These enzymes- aspartate amino transferees' (AST, formerly serum glutamate oxaloacetic trans aminase-SGOT) and alanine amino transferase (ALT, formerly serum glutamic pyruvate transaminase-SGPT) catalyze the transfer of the á amino acids of aspartate and alanine respectively to the á keto group of keto glutaric acid. ALT is primarily localized to the liver but the AST is present in a wide variety of tissues like the heart, skeletal muscle, kidney, brain and liver.

AST: alanine + a ketoglutarate = oxaloacetate + glutamate

ALT: alanine + a ketoglutarate = pyruvate + glutamate.

Whereas the AST is present in both the mitochondria and cytosol of hepatocytes, ALT is localized to the cytosol. The cytosolic and mitochondrial forms of AST are true iso enzymes and immunologically distinct (Flamm *et al.,* 2002).

About 80% of AST activity in human liver is contributed by the mitochondrial isoenzyme, whereas most of the circulating AST activity in normal people is derived from the cytosolic isoenzyme. Large increases in mitochondrial AST occur in serum after extensive tissue necrosis. Because of this, assay of mitochondrial AST has been advocated in myocardial infarction. Mitochondrial AST is also increased in chronic liver disease. Their activity in serum at any moment reflects the relative rate at which

they enter and leave circulation. Of the numerous methods used for measuring their levels, the most specific method couples the formation of pyruvate and oxaloacetate-the products of the amino transferase reactions to their enzymatic reduction to lactate and malate (Rothschild *et al.*, 1995).

MILD, MODERATE AND SEVERE ELEVATIONS OF AMINO TRANSFERASES

1. Severe (> 20 times, 1000 U/L):

The AST and ALT levels are increased to some extent in almost all liver diseases. The highest elevations occur in severe viral hepatitis; drug or toxin induced hepatic necrosis and circulatory shock. Although enzyme levels may reflect the extent of hepatocellular necrosis they do not correlate with eventual outcome. In fact, declining AST and ALT may indicate either recovery of poor prognosis in fulminant hepatic failure (Friedman *et.al,* .2003)

2. Moderate (3-20 times):

The AST and ALT are moderately elevated in acute hepatitis, neonatal hepatitis, chronic hepatitis, autoimmune hepatitis, drug induced hepatitis, alcoholic hepatitis and acute biliary tract obstructions. The ALT is usually more frequently increased as compared to AST except in chronic liver disease. In uncomplicated acute viral hepatitis, the very high initial levels approach normal levels within 5 weeks of onset of illness and normal levels are obtained in 8 weeks in 75% of cases. For reasons, which are not, understood AST levels appear disproportionately low in patients with Wilson disease (Rosalki *et al.,* 1999).

3. Mild (1-3 times):

These elevations are usually seen in sepsis induced neonatal hepatitis, extrahepatic biliary atresia (EHBA), fatty liver, cirrhosis, non-alcoholic steato hepatitis (NASH), drug toxicity, myositis, Duchenne muscular dystrophy and even after vigorous exercise.1,4 One third to one half of healthy individuals with an isolated elevation of ALT on repeated testing have been found to be normal. (Rosalki *et al.*,1991)

Falsely low amino transferes levels:

They have been seen in patients on long term hemodialysis probably secondary to either dialysate or pyridoxine deficiency. Low levels have also been seen in uremia.

1.9 LIVER DISEASES

Although liver disease is stereotypically linked to alcohol or drugs, the truth is that there are over 100 known forms of liver disease caused by a variety of factors and affecting everyone from infants to older adults.

1. Jaundice

This is the yellow pigmentation of the skin, mucous membrane and deeper tissues due to increased bilirubin level in blood. The normal serum bilirubin level is 0.5to 1.5 mg%. When this exceeds 2 mg %, jaundice occurs.

Types and causes of Jaundice: Jaundice is classified into three types namely hemolytic jaundice, hepatocellular jaundice, and obstructive jaundice.

a) Hemolytic Jaundice

Hemolytic jaundice is also called prehepatic jaundice. During this, the excretory function of liver is normal. But there is excessive destruction of red blood cells and thus the bilirubin level in blood is increased the liver cells cannot excrete much bilirubin rapidly. So, it accumulates in the blood resulting in jaundice. In this type of jaundice, the free bilirubin level increases in blood. Increased in formation of urobilinogenin resulting in the excretion of more amount of urobilinogenin urine. Any condition that causes hemolytic anemia can lead to hemolytic jaundice.

b) Hepatocellular Jaundice

The jaundice due to the damage of liver cells is called hepatocellular or hepatic jaundice. It is also called hepatic cholestatic jaundice. Here, bilirubin is conjugated. But the conjugated bilirubin cannot be excreted. So, it returns to the blood. The damage of liver cells occurs because of toxic substances (toxic jaundice) or by infection (infective jaundice). Commonly liver is affected by virus resulting in hepatitis.

c) Obstructive Jaundice

This is otherwise called extra hepatic cholestatic jaundice or post hepatic jaundice. It is due to the obstruction of bile flow at any level of biliary system. The bile cannot be poured into small intestine and bile salts and bile pigments enter the circulation. In this, blood contains more conjugated bilirubin.

2. Cirrhosis

The inflammation and damage of parenchyma of liver is known as cirrhosis of liver. This may result in degeneration of hepatic cells and dysfunction of liver. Cirrhosis is a diffuse, chronic, necrotic (degenerative) liver disorder characterized by

progressive hepatocyte injury followed by regeneration and fibrosis leading to disorganization of lobular architecture, pseudo lobule formation and acquired vascular malformation.

3. Tumors of liver

a) Benign tumors

i) Benign hemangioma

ii) Cysts

b) Malignant tumors

i) Secondary metastasis is the most common tumors. It may be from breast, Lungs and colon

ii) Primary tumors

4. Hepatocellular Failure (Sembulingam *et al.,* 2004): It may occur due to

- Ultra structural lesions of hepatocytes e.g., Raye ‘s syndrome.
- Chronic liver diseases e.g., chronic hepatitis, cirrhosis, Wilson ‘s disease.
- Coma

5. Hepatic Encephalopathy: Also called as hepatic coma, is a feature of chronic liver failure. It is a metabolic disorder of the central nervous system and neuromuscular system associated with hepatic failure. It is reversible condition.

6. Portal Hypertension: In this condition, there is increased resistance to portal blood flow. It may occur in the conditions of Portal vein thrombosis, Splenomegaly, Cirrhosis.

7. Cirrhosis: Cirrhosis is often considered to be a form of liver disease and may be the only liver-related condition that many people have heard of. In fact, cirrhosis is a condition that results from permanent damage or scarring of the liver. It is the end stage of many different forms of liver disease and is known to cause a number of other health problems, including variceal bleeding, ascites and hepatic encephalopathy (Sembulingam.*et.al.* 2004).

8. Hepatitis: Hepatitis is 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 vaccine, there is no vaccine for hepatitis C. In Canada, hepatitis C is the leading cause of liver transplants

1. **Hepatitis A** is the most common and the most infectious, spreading easily from person to person like most other viruses. It affects millions around the world and is responsible for more than 2 million deaths a year.

2. **Hepatitis B** is acquired through exposure to infected blood, vaginal fluids, or semen. It's estimated that about 0.5% to 1% of Canadians have hepatitis B.

3. **Hepatitis C** affects about 3.5 million North Americans. About 15% of those with hepatitis C may have been exposed to infected blood products before widespread blood testing began.

4. **Hepatitis D** is unique because it can only affect those that already have hepatitis B.

9. Alcoholic liver disease is any hepatic manifestation of alcohol overconsumption, including fatty liver disease, alcoholic hepatitis, and cirrhosis. Analogous terms such as "drug-induced" or "toxic" liver disease are also used to refer to the range of disorders caused by various drugs and environmental chemicals.

10. Fatty liver disease (hepatic steatosis) is a reversible condition where large vacuoles of triglyceride fat accumulate in liver cells. Non-alcoholic fatty liver disease is a spectrum of disease associated with obesity and metabolic syndrome, among other causes. Fatty liver may lead to inflammatory disease (i.e., steatohepatitis) and, eventually, cirrhosis.

Hereditary diseases that cause damage to the liver include hemo chromatosis, involving accumulation of iron in the body, and Wilson's disease, which causes the body to retain copper. Liver damage is also a clinical feature of alpha 1-antitrypsin deficiency and glycogen storage disease type II (Bhanwra *et al.*, 2000).

CAUSES OF LIVER DISEASES: In many cases the dysfunction of liver will be secondary to a problem elsewhere in the body such as:

1. **Trauma:** Animals that receive a severe and blunt blow on the abdomen can suffer from liver disease. The most common cause of this type of blow is being hit by a car there by a liver lobe fracture and bleeding into the abdomen, even leading to death and a more common occurrence is a bruise (contusion), Heatstroke, diaphragmatic hernia and liver lobe torsion can also cause liver problems.

2. **Inflammation:** An inflamed condition of liver is called hepatitis. Trauma can cause this, along with other drugs, viruses, bacteria, bile and toxins.
3. **Pancreatitis:** The severe inflammatory process that occurs with digestive enzymes can spill over into the liver and cause severe disease.
4. **Anemia:** Hemolytic anemia can decrease the oxygen availability to liver cells and lead to their death
5. **Infection:** Bacteria, viruses, and fungi can all cause liver disease. Since bacterial infection is common in many liver problems it is routine to use antibiotics when treating liver problems. Specific diseases include infectious canine hepatitis, canine herpes virus, feline infectious peritonitis (FIP), leptospirosis, abscesses, histoplasmosis, coccidiomycosis and toxoplasmosis. Acute viral hepatitis is a systemic infection manifested primarily by an acute attack on the hepatocytes. Five hepatotropic viruses have been identified (HAV, HBV, HCV, HDV, HEV). Hepatitis A (HAV) causes acute, self-limited disease that is transmitted orally. Hepatitis B (HBV) and hepatitis C viruses (HCV) are transmitted by exchange of body fluids such as blood transfusion or sexual contacts. Hepatitis D (HDV) is a viroid that causes inflammation only in concrete with HBV. Hepatitis E (HEV) virus is transmitted by enteric route and cause self-limited diseases. Chronic hepatitis is an uncommon, but important, complication of HBV- HDV infection. The liver injury also results from an inflammatory immune attack against hepatocytes. In drug-induced hepatitis, a number of drugs have been reported, including methyldopa, nitrofurantoin, isoniazid, ketoconazole and acetaminophen (Gennavo *et al., 2000)*.
6. **Toxins:** There are literally thousands of chemicals that could be toxic to the liver and a few examples of these chemicals that are commonly used in the treatment include: (Ishak *et al.,* 2002).

    - Rimadyl (arthritis treatment) in Labradors
    - Thiacetarsamide (heartworm treatment)
    - Ketoconazole (fungal treatment)
    - Tylenol (acetaminophen)
    - Glucocorticoids (cortisone)
    - Anthelmintics (deworming medication)

- Parasiticides

7. **Cancer:** Cancer can arise directly within the liver (primary) or spread from elsewhere (metastatic or secondary) through the circulatory or lymphatic systems. In the anatomy section as mentioned the dual blood supply to the liver; the portal vein and the hepatic artery. This extra blood supply increases the chance that a tumor in a different organ that has spread into the bloodstream will end up in the liver. As mentioned in the physiology section, liver cancer is usually detected only after the disease is well established, since functional reserve capacity allowed the liver to function normally for a prolonged period of time (Zimmerman *et al.*, 2002).

Some of these liver cancers include:

- Lymphosarcoma
- Hemangiosarcoma
- Metastatic:
- Adenocarcinoma
- Mammary tumors
- Oral carcinoma
- Lymphosarcoma
- Hemangiosarcoma

Metabolic diseases that cause secondary liver problems:

- Hypothyroidism
- Hyperthyroidism
- Pancreatitis
- Diabetes mellitus
- Cushing's syndrome
- Inflammatory bowel disease

1.10 TYPES OF LIVER DYSFUNCTION

Most of the clinical consequences of liver disease can be understood either as a failure of one of the liver's four broad functions or as a consequence of portal hypertension, the altered hepatic blood flow of cirrhosis.

a) **Hepatocyte Dysfunction:** One mechanism of liver disease, particularly in acute liver injury, is dysfunction of the individual hepatocytes that make up the liver parenchyma. The pathway and extent of hepatocellular dysfunction determine the specific manifestations of liver disease.

b) **Portal Hypertension:** Of greatest clinical importance are the existence under normal circumstances of a low-pressure portal venous capillary bed throughout the liver parenchyma and the functional zonation of portal blood flow.

When pathologic processes (e.g., fibrosis) result in elevation of the normally low intrahepatic venous pressure, blood backs up and a substantial fraction of it finds alternative routes back to the systemic circulation, bypassing the liver. Thus, blood from the GI tract is, in effect, filtered less efficiently by the liver before entering the systemic circulation. The consequences of this portal-to-systemic shunting are loss of the protective and clearance functions of the liver, functional abnormalities in renal salt and water homeostasis, and a greatly increased risk of GI hemorrhage from the development of engorged blood vessels carrying venous blood bypassing the liver (esophageal varices) (Agarwal *et al.*,1983).

Even in the absence of any intrinsic parenchymal liver disease, portal-to systemic shunting of blood can produce or contribute to encephalopathy (altered mental status resulting from failure to clear poisons absorbed from the GI tract), GI bleeding (resulting from esophageal varices), and malabsorption of fats and fat-soluble vitamins (caused by loss of enterohepatic recirculation of bile), with associated coagulopathy.

Manifestations of Liver Dysfunction

Whether a result of hepatocyte dysfunction or portal-to-systemic shunting, prominent features of liver disease are manifestations of failure of normal functions. An understanding of these mechanisms offers insight into the probable causes of illness in a patient with acute or chronic liver disease.

Mechanism of liver damage:

Liver damage is explained on basis of following mechanisms:

- Alcohol toxicity may be associated with increased oxidative stress and free radical associated injury. Generation of oxygen metabolites such as super oxide (O_2^-) hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^-) is

believed to be important in the pathogenesis of alcoholic liver injury (Davidson *et al.*,1999).

- Acetaldehyde (the major intermediate metabolite of alcohol en route to acetate production) induces lipid per oxidation and acetaldehyde – protein adducts formation, further disrupting cytoskeleton and membrane function.

1.11 CCL_4 INDUCED HEPATOTOXICITY:

Liver injury due to carbon tetrachloride in rats was first reported in193639 and has been widely and successfully used by many investigators. Carbon tetrachloride is metabolized by cytochrome P-450 in endoplasmic reticulum and mitochondria with the formation of CCl3O-, a reactive oxidative free radical, which initiates lipid per oxidation (Hayman *et al.*,1976).

Administration of a single dose of CCl4 to a rat produces, within 24hrs a centrilobular necrosis and fatty changes the poison reaches its maximum concentration in the liver within 3hrs of administration. Thereafter, the level falls and by 24hrs there is no CCl4 left in the liver. The development of necrosis is associated with leakage of hepatic enzymes into serum. Dose of CCl_4: 0.1 to 3 ml/kg I.P (Dawkins *et al.*,1976).

Literature Review

2. LITERATURE REVIEW

S.no	Plant name	Telugu name	Family	Parts used	Chemical constituents	Parameters monitored	References
1.	Aegle marmelos	Maredu /Bilva	Rutaceae	Leaves	Saponins, flavonoids, glycosides, alkaloids and tannins	AST(U/L), ALT(U/L), ALP(U/L), LDH(U/L), total bilirubin(mg/dl), total protein(mg/dl)	D. Rathee *et al*., 2018
2.	Aloe barbadensis mill.	Kala bandha	Liliaceae	Aerial part	Flavanoids, hydroxyanthraquinones and coumarin	Serum bilirubin(mg/dl), serum ALT(IU/L), serum AST(IU/L), Serum ALP(IU/L)	Bhatt *et al*., 2014
3.	Azadirachta indica	Vepa	Meliaceae	Whole parts	Azadirachtin, margolone, mono-, di-, sesqui- and triterpenoids, coumarins, chromones, lignans, flavonoids and other phenolics	AST(U/L), ALT(U/L), ALP(U/L), MDA (nmol/g protein), GSH (µg/mol protein), SOD(U/mg), IL-1 (pg/ml), TNF-α (pg/ml)	Maha A. Althaiban *et al*., 2019
4.	Chamomile capitula	Chamanthi	Compositae	Whole parts	α-bisabolol, α-bisabolol oxide A and B, chamazulene, sesquiterpenes; coumarins: umbelliferone; flavonoids: luteolin,	Blood GSH (mg%), liver GSH (µmoles/g liver), Na-k ATPase (U/mg protein), TBARS (nmol of MDA /g of wet tissue), ALT (U/mg protein),	Ajay Kumar Gupta *et al*., 2006

					apigenin, quercetin and spiroethers: en-yn dicycloether	AST (U/mg protein), ALP (KA unit), bilirubin(mg%), TSP (mg protein/ml serum), GLY (mg/g wet tissue), body weight(g), liver weight(g), before treatment(g), after treatment(g)	
5.	Citrus limon L. Burm	Nimma	Rutaceae	Fruits	Flavonoids like diosmin, hesperidin, limocitrin and phenolic acids like ferulic acid, sinapic acid, p-hydroxy benzoic acid, D-limonene, β-pinene, γ-terpinene	GST (μmole mL^{-1} min^{-1} mg^{-1} protein, SOD liver (one unit $min^{-1}mg^{-1}$), Catalase liver (µ mol $min^{-1}mg^{-1}$), AChE liver (μmole mL^{-1} min^{-1} mg^{-1})	Jaiswal S.K. *et al*., 2015
6.	Curcuma longa	Pasupu	Zingiberaceae	Rhizome	Curcumin, turmerone, monoterpenes,5%curcuminoids, minerals, carotene and vitamin C	Total protein (g/L), Albumin (g/L), TB (µM/L), AP (IU/L), ALT (IU/L), AST (IU/L), GGT (IU/L), Body weight (g) Liver weight (g) Liver weight × 100/body weight	Salama *et al*., 2013
7.	Decalepis hamiltonii (Wight and Arn)	Nannari kommulu	Asclepiadaceae	Root	4-Omethylresorcylaldehyde, benzyl alcohol,	SGOT, SGPT, LDH, ALP and lipid peroxidation and	Harish *et al*.,

					β-caryophyllene and α-atlantone, Aromatic aldehydes, monoterpene, hydrocarbons, alcohols and ketones, β-phellandrene and trans-anethole	glutathione (GSH), catalase, glutathione peroxidase, glutathione reductase, glutathione transferase	2010
8.	Ficus carica	Anjeer	Moraceae	Leaves	phenolic compounds, phytosterols, organic acids, anthocyanin composition, triterpenoids, coumarins, and volatile compounds such as hydrocarbons, aliphatic alcohols	Pentobarbitone sleeping time (min), liver weight (g), SGPT (units/L), SGOT (units/L)	Gond *et al*., 2008
9.	Glycyrrhiza glabra	Yashtimadhukam	Leguminosae	Glycyrrhizin from root	Saponin, flavonoids, alkaloids, steroids, terpenoids, tannins and glycosides, carbohydrates, proteins, phlobatannins and phenolic compounds	SALT (U/L), SAST (U/L), SALP (U/L), TSB (µmol/L), TSP (g/dL)	Al-razzuqi *et al*., 2012
10.	Indigophora tinctorea	Aviri, Kondanili	Fabaceae	Whole plant	Inorganic salts of nitrogen, phosphoric acid, lime, potash along with apigenin, kaempferol, luteolin, quercetin, seed-galactomannan,	AST(U/L), ALT (U/L), ALP (U/L), Total bilirubin (mg%), Total protein (mg%) GGTP (U/L)	Felicia *et al*., 2012

					galactose, mannose		
11.	Lepidium sativum	Aadithyalu, Aandilee	Brassicaceae	Seeds	cardiac glycoside, alkaloids, phenolic, flavonoids, cardiotonic glycosides, coumarins, glucosinolates, carbohydrates, proteins and amino-acids, mucilage, resins, saponins, sterols, tannins, volatile oils, triterpene, sinapic acid and uric acid.	AST, ALT, ALP, (U/L), Bilirubin, plasma(mg/dL), γ - GGT, CAT (u/mg), GSH (nmol/mg) MDA, SOD(u/mg), protein(nmol/min/mg)	Raish *et al*., 2016
12.	Momordica dioica	Akakara	Cucurbitaceae	Leaves	glycosides, flavonoids, triterpenoids, steroids, alkaloids, s, thiamine, A vitamin, acids ascorbic, acids fatty saturated and oil semidrying brown dark acid urisolic of triterpenes spiranosterol-alpha, gypsogenin, acid stearic, acid oleanoic, principles bitter, carotenes, acids ascorbic], 15 [lectins,	T. Bilirubin (mg/dl), SGOT(U/L), SGPT(U/L), ALP(U/L)	Kushwaka s.k *et al*., 2005

					carbohydrates protein, niacin l, hedera		
13.	Moringa oleifera Lam.	Munaga	Moringeaceae	Seed	Hydrocarbons, hexacosane, pentacosane, heptacosane, (E)-phytol, thymol, hexanoic acid, acetic acid, nonacosane, 1,2,4-trimethyl-benzene	ALT (U/L) AST (U/L) ALP (U/L) Albumin (g/dl) T. protein (g/dl) SOD (% inhibition) GSH (mM/g Hb) MDA (µM/ml packed cell) TAC (mM/L) Catalase U/g tissue	Kadry El-bakry *et al*., 2016
14.	Ocimum sanctum	Thulasi	Lamiaceae	Leaves	Alkaloids, tannin, saponin, steroid phlobatannin, terpenoid, flavonoid, cardiac, glyceride	Total protein (g/dl) Albumin globulin ratio Serum ALP (KA units) Serum AST (IU/L) Serum ALT (IU/L)	Lahon, K *et al*., 2011
15.	Phyllanthus emblica	Usiri	Euphorbiaceae	Whole plant	Protein, fats, fibres, carbohydrates, vitamin-C, nicotinic acid, tannins, gallic acid, ellagic acid, flavin and glucose, linolenic acid, oleic acid	TNF-α, IL-1β, and IL-6, SOD, MDA, GSH, PC, NO, LN (ng/ml), HA (ng/ml), IV-C(ng/ml), PC-III (ng/ml)	Yin, K *et al*., 2021
16.	Physalis minima	Kupanti	Solanaceae	Whole plant	cardiac glycosides, phenols, quinones, reducing sugars, saponins, steroids, starch, tannins and terpenoids	SGPT (U/L), SGOT (U/L), Total Bilirubin (mg/ml)	M. Pratheeba *et al*., 2014

17.	Pterocarpus marsupium Roxb.	Yegisa	Papilionaceae	Stem bark	Protein, pentosan, mucilage, pterosupin, pseudobaptigenin, liquiritigenin, garbanzol, beta-cudesmol, pterostilbene, marsupol, carpusin, proterol, marrsupinol, parsupin, oleanolic, tannins and ksinotanic acid, quercetin, kaempferol, epicatechin, and rutin, phytol, 1H-indene, 1-ethylideneoctahydro-7 a-methyl, (1E,3a. alpha.,7a. beta.), 2H-1-Benzopyran,6,7-dimethoxy-2,2-dimethyl, Inositol,1-deoxy, 2-Methoxy-4-vinylphenol, 2-methoxy-3-2-propenylphenol-, 2 Ethylacridine, Delta-selinene and fatty	SGOT, SGPT, SALP and serum bilirubin	Jadhav Anil G. *et al*., 2019

					acids		
18.	Punica granatum	Dhanimma	Punicaceae	Whole plant	Triterpenoids, steroids, glycosides, saponins, alkaloids, flavonoids, tannins, carbohydrates and vitamin C	AST (IU/L), ALT (IU/L), ALP (KAU/dl), Total Bilirubin (mg/dl)	Bushra Hasan Khan *et al*., 2017
19.	Ricinus communis	Aamudham	Euphorbiaceae	Leaves	alkaloids, saponin, tannin, lignin, protein, carbohydrate, suberin, glucoside, oil, sugars, steroids	AST(U/L), ALT (U/L), ALP (U/L), Total bilirubin (mg/dl)	Warda M. A. Kaidama *et al*., 2021
20.	Sesbania grandiflora L.	Avisa	Fabaceae	Whole plant	Sterols, saponins, and tannins	SGPT (u/l) SGOT (u/l) SALP (u/l) Direct bilirubin (mg/dl) Total bilirubin (mg/dl) Total protein (mg/dl) Albumin (mg/dl)	Tathe *et al*., 2010
21.	Solanum nigrum	Kamanchi	Solanaceae	Fruits, leaves	Steroidal components, withanolides, Flavonoids, terpenoids	AST (mU/mg protein), ALT (mU/mg protein), ALP (µmol/mg protein/30min), ACP (µmol/mg protein/30min), DNA (µmoles/100mg tissue weight), RNA (µmoles/100mg tissue weight),	Krithika R *et al*., 2019

						protein (mg/100mg tissue weight), SDH, ATPase.	
22.	Trigonella foenumgraecum	Menthulu	Fabaceae	Leaves, seeds	Fibers, flavonoids, polysaccharides, saponins, flavonoids and polysaccharides fixed oils alkaloids	SGOT (IU/L) SGPT (IU/L) SALP (IU/L) Total cholesterol (mg/dL) HDL (mg/dL) LDH (IU/L)	Das S *et al*., 2014
23.	Vitis vinifera	Draksha	Vitaceae	Leaves	Phenolic acids, flavonoids, anthocyanins, proanthocyanidins, sugars, sterols, amino acids and minerals	AST(IU/L) ALT(IU/L) LDH(IU/L) SALP (mg/Pi /h/100ml) Bilirubin(mg/dl), Albumin (g/dl)	Sharma *et al*., 2021
24.	Wedelia calendulacea	Pilabhangara/ yellow bhringraj	Asteraceae	Whole plant	isoflavonoids, bisdesmoside, oleanolic acid saponin and wedelolactone (analogous to coumestrol an estrogen from clover), carotene, chlorophyll, tannin, saponin, phytosterol, waxy compound and resin.	Bilirubin(mg/dl), AST(U/L), ALT(U/L), ALP(U/L), protein (g/dl	Murugain P *et al*., 2008
25.	Zingiber officinale Ros.	Allam	Zingiberaceae	Rhizome	Fibres, proteins, starch, carbohydrates, resin, glutamine, thrionin, free aminoacid,	Total bilirubin (mg/ dl), Triglyceride (mg/dl), Cholesterol (mg/dl), Total protein	Atta *et al*., 2010

					zingiberol, zingiberin, glutamic acid, aspartic acid	(g/dl), Albumin (g/dl),	

Aim & Objective

3. AIM & OBJECTIVE

Aim:

The main aim of the study was to evaluate the hepatoprotective activity of leaves of *Wedelia calendulacea* in validated experimental animal models.

Objective:

The objective of the entitled project is to carry out and evaluate the following parameters:

- **Collection, identification and authentication of plant material**
- **Extraction**
  - Soxhlet extraction
- **Preliminary phytochemical analysis**
- **Hepatoprotective activity**
  - Acute hepatotoxicity studies
    - Carbon tetrachloride induced hepatotoxicity
  - Estimation of serum biochemical parameters
    - Total protein
    - Aspartate aminotransferase (AST)
    - Alanine aminotransferase (ALT)
    - Serum albumin
    - Serum creatinine

Plant profile

4. PLANT PROFILE

Fig 6: Plant of *Wedelia calendulacea*

4.1 TAXONOMY:

Plant name : *Wedelia calendulacea*

Family : Asteraceae

Synonyms : Sphagneticola calendulacea, Wedelia chinensis

Common names : Bay Biscayne creeping-oxeye, Singapore daisy, creeping-oxeye, trailing daisy, and wedelia

4.2 VERNACULAR NAMES:

English : Chinese Wedelia

Hindi : Pilabhangara, Bhanra marathi

Sanskrit : Pitabhrnga, Pitabhrngarajah

Tamil : Manjalkarilamkanni

Kannada : Gargari, Kalsargi

4.3 GEOGRAPHICAL DISTRIBUTION

Wedelia calendulacea is a perennial herb that grows in wet places in Uttar Pradesh, Assam, Arunachal pradesh and Tamil Nadu (Hone C *et al*., 1992)

4.4 BOTANICAL DESCRIPTION:

Wedelia calendulacea (Asteraceae) is a perennial herb of about 0.3 to 0.9 cm height. Leaves are fleshy, usually 4-9 cm long and 2-5 cm wide, irregularly toothed or serrate, usually with a pair of lateral lobes and obviate in shape. Flowers are yellow, tubular in terminal or axillary head and 4-5 cm in diameter (Umashankar *et al*., 2010). It is scabrous procumbent perennial soft herb with high camphor like odour and has a gorgeous growth. It is a perennial herb of 0.3-0.9 m high, stem procumbent at base and rooting at lower nodes. Leaves are opposite, subsessile,2.5-7.5 by 1-2.8 cm lanceolate-oblong, entire or irregularly cenate-serate, scabrous with short white hairs and base tapering. Heads of flowers, 2-3.2 cm diameter, solitary, peduncles 2.5-15cm long erect, slender, slightly thickened beneath the heads (Koul *et al*., 2012).

4.5 ACTIVE CONSTITUENTS:

The plant contains alkaloids, saponins, tannin, flavonoids, a lactone, wedelolactone and norwedilic acid. Expressed juice of *Wedelia calendulacea* contains an oil-soluble black dye, waxy compounds, phytosterols, carotene and resin. The plant also contains inorganic salts, siliceous materials, pectin and mucin (Ghani *et al*., 2003). The leaves contain isoflavonoids, bisdesmosidic oleanolic acid saponins and wedelolactones, nor wedelolactone has also been isolated from alcoholic extract of leaves.

4.6 ETHNO MEDICINAL USES:

The plant has traditional uses which include kidney dysfunction, cold, wounds healing, amenorrhea, dying hair, antioxidant, skin disease, cephalalgia, alopecia, inflammatory, multiple sclerosis, antifungal, mental tension, antimicrobial, antistress, anticandidal, jaundice. (Shamama *et al*., 2017).

Table 1: Earlier research works done on *Wedelia calendulacea*

S. No	Earlier works done	Reference
1.	Umasankar K et al., 2010 studied the CNS Activity of Ethanol Extract of *Wedelia calendulacea* in Experimental Animals. rats. The CNS effects were evaluated by general behavior, exploratory behavior, muscle relaxant activity and phenobarbitone sodium–induced sleeping time using standard procedures in experimental animal models.	Umasankar K *et al*., 2010.
2.	Mishra G et al., 2009 have done their work on Hepatoprotective activity of alcoholic and aqueous extracts of *Wedelia calendulacea* by estimating the serum transaminases (SGOT, SGPT), Serum alkaline phosphatase (SALP), total and direct bilirubin and liver weight of albino rat. The present study revealed that among the two extracts tested, alcoholic extract at a dose level of 500 mg/kg was found to possess significant protective effect against hepatotoxicity	Mishra G *et al*., 2009.
3.	Emmanuel S et al., 2001 studied the Hepatoprotective effect of coumestans isolated from the leaves of *Wedelia calendulacea* Less. in paracetamol induced liver damage	Emmanuel S *et al*., 2001.
4.	Lin, F.M et al., 2007 reported Compounds from *Wedelia calendulacea* synergistically suppress androgen activity and growth in prostate cancer cells	Lin, F.M *et al*., 2007.

Materials & Methods

5. MATERIALS & METHODS

5.1 MATERIALS

Selection of plant material

The selections of plant were made on the basis of:

- Ethno medical information
- Easy availability.
- Cost-effectiveness.
- Method of preparation.
- Therapeutic value.

Chemicals

All the chemicals used in the study were of analytical grade. The following chemicals were used for the experimental study.

Table 2: List of chemicals used

Chemical	Company
Acetic anhydride	Sigma chemicals
Agar	S.d. fine chemicals Ltd
Alcohol	S.d. fine chemicals Ltd
Alcoholic potassium hydroxide	Sigma chemicals
Alfa naphthol	Sigma chemicals
Ammonia	Sigma chemicals
Ammonium hydroxide	Sigma chemicals
Barfoeds reagent	S.d. fine chemicals Ltd
Benedict's reagent	S.d. fine chemicals Ltd
Casein hydrolysate of soya bean	S.d. fine chemicals Ltd
Chloroform	S.d. fine chemicals Ltd
Copper sulphate	S.d. fine chemicals Ltd
Dextrose	S.d. fine chemicals Ltd
Dilute hydrochloric acid	S.d. fine chemicals Ltd
Dragendroff reagent	S.d. fine chemicals Ltd
Ethanol	S.d. fine chemicals Ltd
Fehling's solution A	S.d. fine chemicals Ltd
Fehling's solution B	S.d. fine chemicals Ltd
Ferric chloride	S.d. fine chemicals Ltd
Hager's reagent	S.d. fine chemicals Ltd
Magnesium turnings	Bros scientifics
Mayer's reagent	S.d. fine chemicals Ltd
Millons reagent	S.d. fine chemicals Ltd

Ninhydrin reagent	S.d. fine chemicals Ltd
Nitric acid	S.d. fine chemicals Ltd
Petroleum ether	S.d. fine chemicals Ltd
Phenolphthalein	S.d. fine chemicals Ltd
Potassium dichromate	S.d. fine chemicals Ltd
Pyridine	Bros scientifics
Sodium chloride	S.d. fine chemicals Ltd
Sodium hydroxide	S.d. fine chemicals Ltd
Sodium picrate	S.d. fine chemicals Ltd
Sodium nitroprusside	S.d. fine chemicals Ltd
Sulphuric acid	S.d. fine chemicals Ltd

Animals

Male Wistar albino rats weighed about 150-200 gm were used for the study. The animals were housed in groups of six and maintained under standard conditions (27±2ºC, relative humidity 44 - 56% and light and dark cycles of 10 and 14 hours, respectively) and fed with standard rat diet and purified drinking water and libitum for 1 week before and during the experiments.

All experiments and protocols described in the present study were approved by the Institutional Animal Ethical Committee (IAEC) and with permission from Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

Instruments

Table 3: List of equipments used

S.no	Instruments	Manufactured company
1	UV-Visible Spectrophotometer	UV-1800 Shimadzu, Model, Mfg by Shimadzu Corporation.
2	Centrifuge	Research centrifuge, Mfg by Remi Instruments Ltd, Mumbai
3	Tissue Homogenizer	Type: RO-127A, Mfg by Rajendra Elect, IND. Ltd, Remi Instruments Division, Vasai
4	Sonicator	Pci made in Mumbai.
5	Milli pore water collector	Mfg by TKA smart pure made in made in Germany
6	Soxhlet apparatus	Agarwal

7	Rotary evaporator	Medika instrument Mfg Co.
8	UV chamber	Singhla sciences, Ambala

Plant Material:

Collection and authentication of plant material:

The leaves of *Wedelia calendulacea* were collected from tirumala hills and authenticated by the botanist Dr. Madhava Chettty, Assistant Professor, Dept of Botany, Sri Venkateswara University, Tirupati. The voucher specimen of the plant was deposited at the college for further reference.

Fig 7: collected leaves of the plant

Fig 8: dried leaves of the plant

5.2 METHODS

5.2.1 Preparation of *Wedelia calendulacea* leaf extract:

Soxhlation process:

First method:

for extraction, about 50g of the shade dried and powdered leaf material was taken. The powdered material was transferred into 250ml quick fit flask and extracted in the soxhlet extractor for 48h using organic solvent namely ethanol. The extract was filtered over whatman filter paper no. 1and the filtrate was concentrated under reduced pressure to pasty mass

Second method

Leaves of *Wedelia calendulacea* were collected and subjected to shade drying. Dried samples were completely dried in an incubator at 60°C for 1hr & then the dried leaves were made into fine powder in mechanical grinder. 50g of powdered leaf sample of plant was taken in a thimble and was subjected to extraction with solvent ethanol. 500ml of Solvent was taken into RBF & extraction was carried out soxhlet. Reflux was continued till solvent became Colourless. Extract obtained was concentrated in rotavapour.

Fig 9: soxhlation of the dried powder

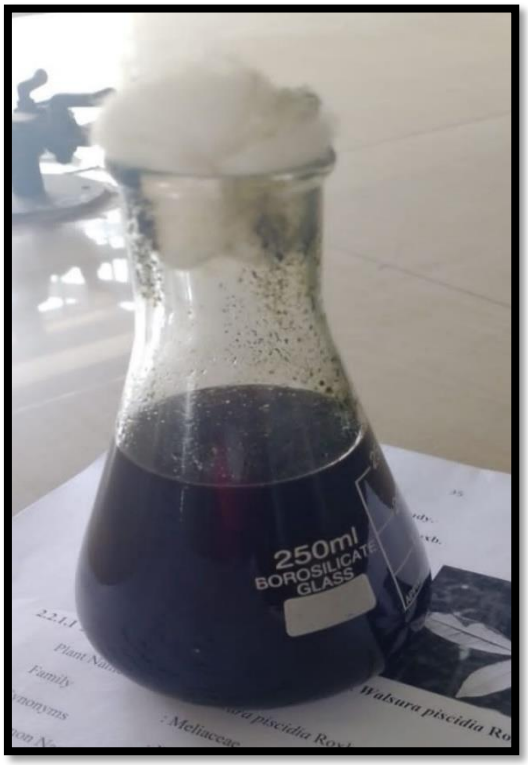


Fig 10: extract collected after soxhlation

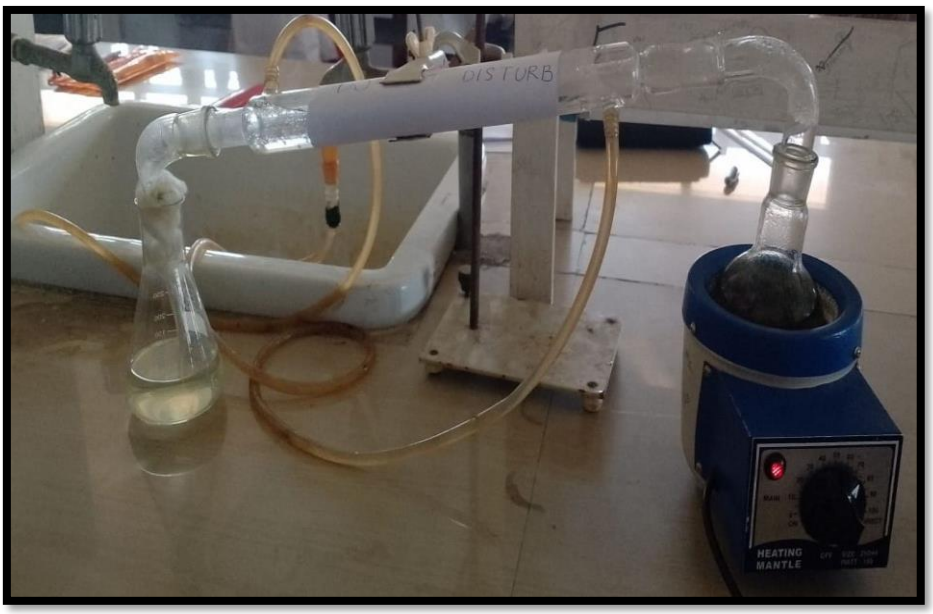


Fig11: reflux condensation for solvent recovery

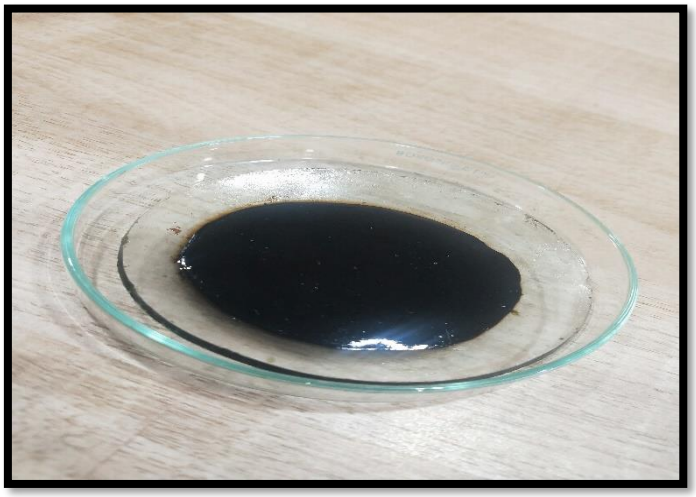


Fig 12: dried extract without solvent

5.2.2 Phytochemical Screening (K. R. Khandelwal 1998)

The extract was then subjected to the Preliminary Phytochemical screening.

1. Test for Carbohydrates:

a. **Molisch's test**: To 2ml of test solution, add alcoholic α-naphthol and then add a few drops of Conc. Sulphuric acid through sides of the test tube. Formation of purple to violet colored ring at the junction confirmed the presence of carbohydrates (reducing sugar).
b. **Barfoed's test:** To 1ml of test solution, add 1ml of Barfoed's reagent and heat on a water bath, the formation of cupric acid gives a green color confirmed the presence of the monosaccharide (reducing sugar).
c. **Fehling's test**: To 1ml of test solution, add 1ml of Fehling's A and B and heat to water bath, the formation of brick red precipitate confirms the presence of the carbohydrate (reducing sugar).
d. **Benedict's test:** To 2ml of test solution, add 1ml of Benedict's reagent and heat on a water bath, the formation of reddish-brown precipitate confirms the presence of the carbohydrates (reducing sugar).

2. Test for alkaloids:

a. **Dragendorff's reagent test:** To 1ml of test solution, add dragendroff's reagent (potassium bismuth iodide). Formation of a reddish-brown precipitate confirms the presence of the alkaloids.

b. **Mayer's reagent test:** To 1ml of test solution, add Mayer's reagent (potassium mercuric iodide solution). Formation of a cream color precipitate confirms the presence of the alkaloids.

c. **Hager's reagent test:** To 1ml of test solution, add Hager's reagent (saturated solution of picric acid). Formation of a yellow color precipitate confirms the presence of the alkaloids.

d. **Wagner's reagent test:** To 1ml of test solution, add Wagner's reagent (iodine-potassium iodide solution). Formation of a reddish-brown precipitate confirms the presence of the alkaloids.

e. **Tannic acid test:** To 1ml of test solution, add tannic acid solution. Formation of buff colored precipitate confirms the presence of the alkaloids.

3. Test for glycosides:

- **Cardiac glycosides:**

a. **Baljet's test**: Treat 2 ml of test solution with picric acid or sodium picrate. Formation of orange color confirms the presence of cardiac glycosides.

b. **Keller-killiani test** (test for deoxy sugars): Extract the drug with chloroform and evaporated to dryness. Add 0.4 ml of glacial acetic acid containing trace amount of $FeCl_3$. Transfer the solution to a small test tube-acetic acid layer shows a blue color if cardiac glycosides were present.

- **Saponins glycosides:**

Foam test: Place 2 ml of drug in water in test tubes; shake well, stable froth forms if saponin glycosides were present.

- **Anthraquinone glycosides:**

Borntrager's test: Boil the test material with 1 ml of H_2SO_4 in a test tube for 5 min. Filter while hot. Cool the filtrate and shake with equal volume of dichloromethane or $CHCl_3$. Separate the lower layer of dichloromethane or $CHCl_3$ and shake it with half of its volume of Dil. NH_3. A rose pink to red color will produce in ammoniacal layer if Anthraquinone glycosides were present.

4. Test for flavonoids:

a. **Shinoda's test**: To the 2ml of test solution, add few magnesium turnings and add conc. HCl drops wise. Pink, crimson red, green to blue color will appear after a few minutes if Flavanoids were present.
b. **Alkaline reagent test:** To the test solution add a few drops of sodium hydroxide solution. Intense yellow color is formed which turns to colorless on addition of a few drops of dilute acid indicate presence of Flavanoids.
c. **Zinc hydrochloride test:** To the test solution add a mixture of zinc dust and conc. Hydrochloric acid. It gives red color after a few minutes if Flavanoids were present.

5. Test for steroids and Tri-terpenoids:

a. **Salkowski test:** Treat the extract with a few drops of conc. Sulphuric acid, the formation of red color at lower layer indicates the presence of steroids or the formation of yellow color at a lower layer indicates the presence of tri-terpenoids.
b. **Libermann-buchard test:** Treat the extract with a few drops of acetic anhydride, boil and cool. Add conc.H_2SO_4 through the sides of the test tube. The formation of the brown color ring at the junction between two layers and the turning of upper layer into green color confirms the presence of steroids and the presence of deep red color indicates presence of tri-terpenoids.

6. Test for tannins and phenolic compounds:

a. **$FeCl_3$ test:** Treat the extract with $FeCl_3$ solution, formation of blue color confirms the presence of hydrolysable tannins and formation of green colour confirms the presence of condensed tannins.
b. **Acetic acid test:** Treat the extract with acetic acid solution, formation of red color confirms the presence of tannins.
c. **Dilute nitric acid test:** Treat the extract with dilute nitric acid solution; formation of red to yellow color confirms the presence of tannins

7. Test for Tannins

Accurately take 1ml of extract solution to that add few drops of water and heat the solution after it filtrate. To that filtrate solution add Ferric Chloride it appears the green colour ppt, it indicates that presence of Tannins.

8. Tests for Phenols

Accurately take few ml of extract to that add 1ml of 5% of Ferric Chloride solution it turns to deep blue or black colour, it indicates that the presence of Phenols.

9. Test for Proteins and amino acids:

a. **Biuret test:** To 1ml of test solution, add 2ml of Biuret reagent and heat on a water bath, the formation of violet colour confirms the presence of the proteins.
b. **Million's test:** To 1ml of test solution, add 2ml of millions reagent and heat on a water bath, the formation of a white precipitate confirms the presence of the proteins.
c. **Ninhydrin test:** To 1ml of test solution, add 2ml of ninhydrin reagent and heat on a water bath, the formation of violet color confirms the presence of the proteins.

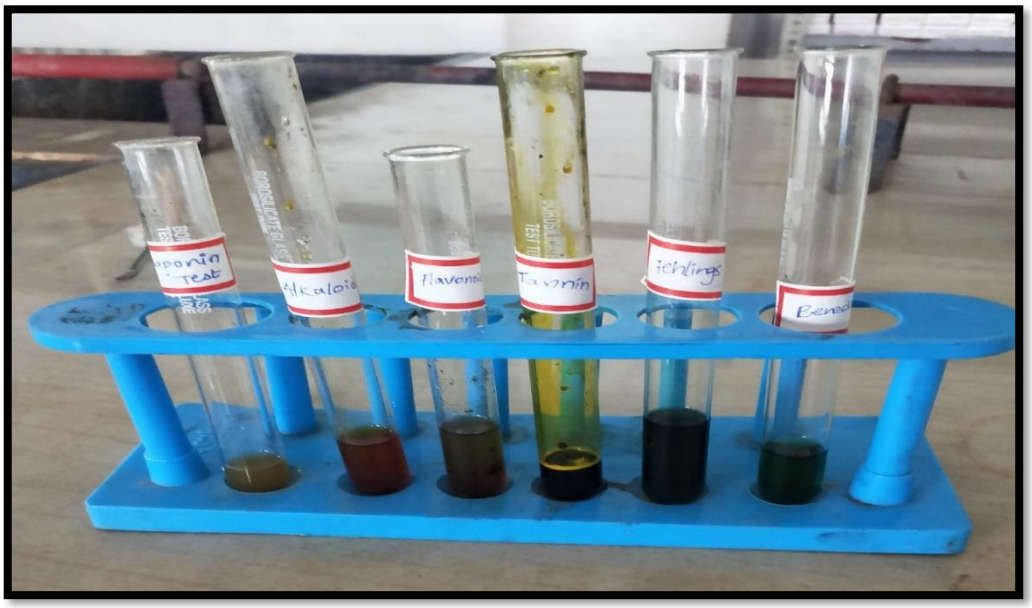


Fig 13: phytochemical analysis of the extract

5.2.3 Hepatoprotective studies

- **Acute hepatotoxicity studies**

Carbon tetrachloride (CCL_4) induced hepatotoxicity (Sangameswaran B et al 2008) Rats of male sex were divided into five groups of six animals in each group (n=6)

Group I: This group received 0.2% of Carboxy methyl cellulose (1ml/kg) once daily for nine days.

Group II: Received 0.2% of Carboxy methyl cellulose solution (1ml/kg) once daily for nine days and carbon tetrachloride (1 ml/kg in 50% v/v olive oil, 1.c.) on the 7th day.

Group III: Received standard drug silymarin (25 mg/kg bwt po.) for 9 days once daily and carbon tetrachloride (1 ml/kg in 50% w/v olive off, sc.) on the 7th day

Group IV. This group received EEWC (200mg/kg bwt po) for 9 days once daily and carbon tetrachloride (1 ml/kg in50% w/v olive oil, S.C.) on the 7th day.

Group V. This group received EEWC (400mg/kg bwt po) for 9 days once daily carbon tetrachloride (1 ml/kg in 50% v/v olive oil, sc.) on the 7" day. On the last day, serum marker enzyme parameters i.e., Serum glutamic pyruvate transaminase (SGPT), Serum glutamic oxaloacetic transaminase (SGOT) (Reitman S 1957) and biochemical parameters Alkaline phosphatase (ALP) (Kind PRN, 1954) and biochemical parameters i.e., Total bilirubin and Total protein were analyzed according to the reported methods.

- **Serum biochemical methods**

1. Estimation of Aspartate amino transferase (AST) or (SGOT)

Principle

- SGOT catalyzes the following reaction, i.e., the transfer of an amino group from L-Aspartate to α-ketoglutarate.
- α- Ketoglutarate + L- Aspartate ↔ L- Glutamate + Oxalo-acetate
- Oxaloacetate so formed is coupled with 2, 4-Dinitrophenyl hydrazine (2,4-DNPH) to give the corresponding hydration which gives a brown color in alkaline medium and this can be measured calorimetrically.

Reagents

Reagent 1: Buffered Aspartate α-KG substrate pH: 7.4.

Reagent 2: DNPH color reagent.

Reagent 3: Sodium hydroxide, 4 N.

Reagent 4: Working Oxaloacetate standard, 2 mm.

Solution 1: Dilute 1 ml of Reagent 3 to 10 ml with purified water.

Procedure

Standard curve

- As the reaction proceeds with time more amounts of products are formed and since the end products inhibit the enzyme, there is more of inhibition. This is the major problem with calorimetric methods for the estimation of this enzyme. On the other hand, in kinetic methods, since the enzyme activity is measured in the initial few minutes, the number of products formed during the short time are negligible to cause any inhibition. Because of the above problem, it is necessary to standardize any calorimetric method against a standard kinetic method.
- The standard graph is drawn with enzyme activity (IU/l) on the x-axis and O.D on y-axis and is not a linear one, which shows that O.D increases with increase in enzyme activity at a decreasing rate.

Table 4: Procedure of SGOT for Standard

Tube No.	**1**	**2**	**3**	**4**	**5**
Assigned enzyme activity (IU/l)	0	24	61	114	190
Reagent to be pipette	Volume in ml				
Reagent 1	0.5	0.45	0.4	0.35	0.3
Reagent 4	-	0.05	0.1	0.15	0.2
Purified water	0.1	0.1	0.1	0.1	0.1
Reagent 2	0.5	0.5	0.5	0.5	0.5
Mix well and allow to stand at room temperature (15 – 30 º C) for 20 minutes					
Solution 1	5.0	5.0	5.0	5.0	5.0

Mix well by inversion. Allow to stand at room temperature (15-30º C) for 10 minutes and measure the O.D of all the five test tubes against purified water on a colorimeter with a green filter at 505 nm.

Table 5: Procedure of SGOT for Test

Pipette into tube marked for test	Test (T)
Reagent 1	0.25 ml
Incubate at 37 ºC for 5 minutes	
Serum	0.05 ml
Mix well and incubate at 37 ºC for 60 minutes	
Reagent 2	0.25 ml
Mix well and allow to stand at room temperature for 20 minutes	
Solution 1	2.5 ml

Mix well by inversion. Allow to stand at room temperature (15-30 º c) for 10 minutes and measure the O.D of all the five test tubes against purified water on a colorimeter with a green filter at 505 nm.

Calculations

Mark the O.D of test on the Y-axis of the standard curve and extrapolate it to the corresponding enzyme activity on X-axis.

2. Estimation of Alanine amino Transferase (ALT) or SGPT

Principle

SGPT catalyzes the following reaction, i.e., the transfer of an amino group from L-Alanine to α-ketoglutarate.

α- Ketoglutarate + L- Alanine ↔ L- Glutamate + Pyruvate

Pyruvate so formed is coupled with 2,4-Dinitrophenyl hydrazine (2,4-DNPH) to give the corresponding hydration which gives a brown color in alkaline medium and this can be measured calorimetrically.

Reagents

Reagent 1: Buffered Alanine α-KG substrate pH: 7.4.

Reagent 2: DNPH color reagent.

Reagent 3: Sodium hydroxide, 4 N.

Reagent 4: Working Pyruvate standard, 2 mm.

Solution 1: Dilute 1 ml of Reagent 3 to 10 ml with purified water.

Procedure

Standard curve

As the reaction proceeds with time, more amounts of products are formed and since the end products inhibit the enzyme, there is more of inhibition. This is the major problem with calorimetric methods for the estimation of this enzyme. On the other hand, in kinetic methods, since the enzyme activity is measured in the initial few minutes, the number of products formed during the short time are negligible to cause any inhibition. Because of the above problem, it is necessary to standardize any calorimetric method against a standard kinetic method.

The standard graph is drawn with enzyme activity (IU/L) on the X-axis and O.D on Y-axis and is not a linear one, which shows that O.D increases with increase in enzyme activity at a decreasing rate.

Table 6: Procedure of SGPT for Standard

Tube No.	1	2	3	4	5
Assigned enzyme activity (IU/L)	0	28	57	97	150
Reagent to be pipette	Necessary Volume in ml				
Reagent 1	0.5	0.45	0.4	0.35	0.3
Reagent 4	-	0.05	0.1	0.15	0.2
Purified water	0.1	0.1	0.1	0.1	0.1
Reagent 2	0.5	0.5	0.5	0.5	0.5
Mix well and allow to stand at room temperature (15-30 º C) for 20 minutes					
Solution 1	5.0	5.0	5.0	5.0	5.0

Mix well by inversion. Allow to stand at room temperature (15-30º C) for 10 minutes and measure the O.D of all the five test tubes against purified water on a colorimeter with a green filter at 505 nm.

Table 7: Procedure of SGPT for Test

Pipette into tube marked for test	Test (T)
Reagent 1	0.25 ml
Incubate at 37 ºC for 5 minutes	
Serum	0.05 ml
Mix well and incubate at 37 ºC for 30 minutes	
Reagent 2	0.25 ml
Mix well and allow to stand at room temperature for 20 minutes	
Solution 1	2.5 ml

Mix well by inversion. Allow to stand at room temperature (15-30º C) for 10 minutes and measure the O.D of all the five test tubes against purified water on a colorimeter with a green filter at 505 nm.

Calculations

Mark the O.D of test on the y-axis of the standard curve and extrapolate it to the corresponding enzyme activity on x-axis.

3. Estimation of total protein (TP)

Principle

Among the various methods available for the quantitative estimation of proteins such as salt fractionation, electrophoresis, ultra centrifugation, etc. Kjeldahl's method is the considered as the reference method. However, this method is time consuming and cumbersome. The proteins bind with copper ions in the alkaline media of Biuret reagent to produce a purple-colored complex whose observance is proportional to the protein concentration.

Reagents

Reagent 1: Biuret reagent.

Reagent 2: Standard.

Procedure

Pipette into clean, dry test tubes labeled Blank (B), Standard (S), and Test (T).

Table 8: Procedure for Total protein

	B	S	T
Biuret Reagent	1.0 ml	1.0 ml	1.0 ml
Distilled water	2.0 ml	2.0 ml	2.0 ml
Standard	-	0.05 ml	-
Serum	-	-	0.05 ml

Mix well and incubate at 37º C for 10 minutes. Measure the absorbance of Standard (S), and Test (T) against Blank (B) with yellow-green filter on a colorimeter at 555 nm.

4. Estimation of creatinine

Turn on the spectrophotometer and let warm up for at least 15 minutes. Set the wavelength to 520 nm. Dilute urine specimens 1:10 with distilled deionized water. Precipitate the proteins present in the patient serum or urine specimens and in each control by adding 0.5 ml of the specimen to 4.0 ml tungstic acid in a test tube. Shake vigorously and centrifuge for 10 minutes. Label cuvettes 1 through 10. Add 1.0 ml of picric acid solution to cuvettes 1 through 10. Add 3.0 ml distilled deionized water to cuvette

Procedure

- Add 3.0 ml of the 0.5 mg/dl creatinine standard to cuvette
- Add 3.0 ml of the 1.0 mg/dl creatinine standard to cuvette
- Add 3.0 ml of the 2.0 mg/dl creatinine standard to cuvette
- Add 3.0 ml of the 4.0 mg/dl creatinine standard to cuvette
- Add 3.0 ml of the 10.0 mg/dl creatinine standard to cuvette
- Add 3.0 ml of the protein free centrifugate of control Level One to cuvette
- Add 3.0 ml of the protein free centrifugate of control Level Two to cuvette
- Add 3.0 ml of the patient's protein free serum or urine centrifugate to the remaining cuvettes.
- Mix by inversion using a paraffin square to prevent spillage.

- Add 0.5 ml of the NaOH solution to the first cuvette. Mix and set a timer for 15 minutes.
- Add 0.5 ml of the NaOH to the remaining cuvettes at 30 second intervals.
- After 15 minutes, place cuvette 1 in the spectrophotometer and set the absorbance to read 0.000.
- Read Absorbance at exactly 15 minutes after adding the NaOH and record the absorbance for cuvettes 2-10.

5. Estimation of Albumin

Principle

Albumin is bound by the BCG dye to procedure an increase in the blue-green colour measured at 630 nm. The colour increase is proportional to the concentration of albumin present

Reagents

Bromocresol Green (BCG) 0.15 g/L, Buffer, pH 4.66± 0.1, surfactant, non-reactive ingredients and stabilizers

Materials Provided

Albumin reagent.

Materials Required

1. Accurate pipetting devices

2. Test tubes/rack

3. Timer

4. Spectrophotometer able to read at 630 nm.

Procedure (Automated)

Refer to specific instrument application instructions.

Procedure (Manual)

1. Label test tubes blank, standard, control, test, etc.

2. Pipette 1.0 ml of reagent into each tube.
3. Transfer 0.01 (10ul) of sample to respective tubes and mix.
4. Incubate all tubes at room temperature for one minute.
5. Zero spectrophotometer with the blank at 630 nm.
6. Read and record absorbance's of all tubes. *For spectrophotometers requiring greater than 1.0 ml to read, 3.0 ml of reagent and 20ul of serum should be used

Statistical analysis

All the data were expressed as mean ± SEM. Statistical significance between more than two groups was tested using one way ANOVA followed by the Tukey's Multiple Comparison test using a computer-based fitness program (Prism, Graph pad.). Statistical significance was determined at $P < 0.001$.


Results

6. RESULTS

Extraction of plant material

The powdered leaf part of plant material was subjected to soxhlet extraction using solvent ethanol. The colour, consistency and yield of ethanolic extract is depicted in table 9.

Table 9: Colour, consistence and yield of the extract

Extract	Colour	Consistence	%Yield (W/W)
Ethanol	Greenish brown	Sticky	17.58%

Preliminary phytochemical screening

The phytochemical studies when brought to little existence, revealed the presence of phytoconstituents like saponin glycosides, steroids, flavonoids, carbohydrates, proteins. The ethanolic extract of leaf part was subjected to different chemical tests separately for the identification of various active constituents was tabulated in the table no: 10.

Table 10: Qualitative Phytochemical analysis of *Wedelia calendulacea*

S.No.	Constituents	Test	Ethanolic Extract
1.	Alkaloids	a) Mayer's reagent b) Dragendorff's reagent c) Hager's reagent d) Wagner’s reagent	+ve +ve +ve +ve
2.	Carbohydrates	a) Molisch's reagent b) Fehling's solution A and B c) Benedict's reagent d) Barfoed's reagent	-ve -ve -ve -ve
3.	Protein	a) Biuret test b) Millon's reagent	-ve -ve
4.	Steroids	a) Liebermann Burchard test b) 5% potassium hydroxide c)Sulphur powder test	+ve +ve +ve
5.	Phenols	a) Ferric chloride b) 10% Sodium chloride	+ve +ve
6.	Tannins	a) 10% Lead acetate solution	+ve

		b) Acetic acid solution c) Aqueous bromine solution	+ve +ve
7.	Flavanoids	a) Alkaline reagent test b) Con. H_2SO_4	+ve +ve
8.	Gums and Mucilage	Swelling test	-ve
9.	Glycosides	Glacial acetic acid + Ferric chloride + Con. Sulphuric acid	+ve
11.	Saponins	Foam test	-ve

Preclinical Toxicity:

Acute oral toxicity study

There was no treatment related death or signs of toxicity developed in the control, and EEWC treated rats throughout the study. Rubbing of nose and mouth on the floor of the cage and restlessness were the only behavioral signs of toxicity shown by the animals and these disappeared with in 24 hrs. of extract administration. During the study there were no significant changes in body weights of treated rats compared to control group. Further there were no gross pathological abnormalities in both control and treated rats. Thus, the LD_{50} value was found to be greater than 2000mg/kg b.wt. with reference to the Globally harmonized system of classification and labelling the chemicals, *Wedelia calendulacea* can be classified as Category -5 and this provides the relevance for protecting human and animal health.

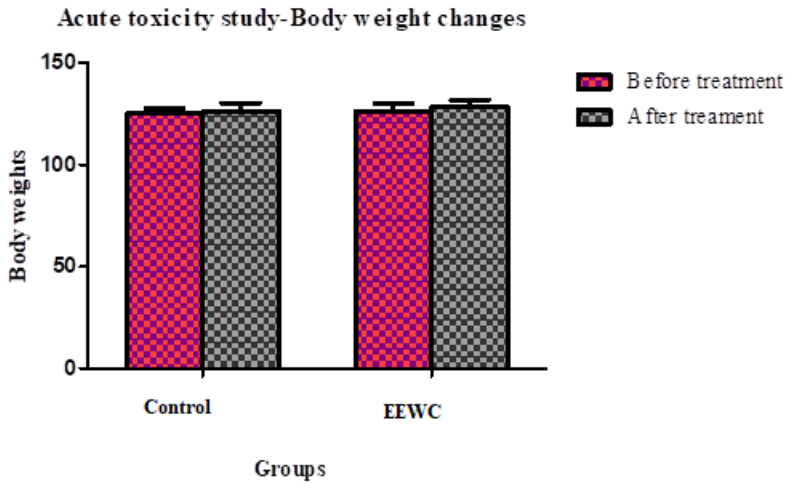


Figure 14: Body weight changes during acute toxicity study

Table 11: Observational changes in rats after oral administration of 2000 mg of EEWC

Observation	30 min		4 hours		24 hours		48 hours		1 week		2 weeks	
	C	EE	C	EE	C	EE	C	EE	C	EE	C	EE
Skin & Fur	N	N	N	N	N	N	N	N	N	N	N	N
Eyes	N	N	N	N	N	N	N	N	N	N	N	N
Mucous membrane	N	N	N	N	N	N	N	N	N	N	N	N
Salivation	N	N	N	N	N	N	N	N	N	N	N	N
Lethargy	A	A	A	A	A	A	A	A	A	A	A	A
Sleep	N	N	N	N	N	N	N	N	N	N	N	N
Coma	A	A	A	A	A	A	A	A	A	A	A	A
Convulsion	A	A	A	A	A	A	A	A	A	A	A	A
Tremors	A	A	A	A	A	A	A	A	A	A	A	A
Diarrhea	A	A	A	A	A	A	A	A	A	A	A	A
Mortality	A	A	A	A	A	A	A	A	A	A	A	A

A indicates absent and N indicates normal

Hepatoprotective activity

Carbon tetrachloride induced toxicity

Effect of EEWC on biochemical parameters in carbon tetrachloride induced hepatotoxic rats.

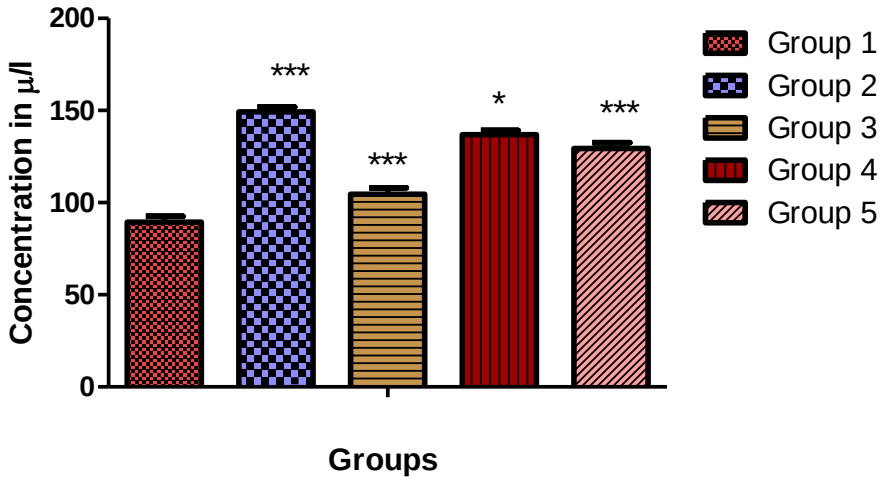
Rats treated with carbon tetrachloride developed a significant hepatic damage observed as elevated serum levels of hepato-specific enzymes like SGPT, SGOT and albumin, total protein and creatinine when compared to normal control. Pre-treatment with Silymarin and EEWC had showed good protection against chloroform induced toxicity to liver. Test group indicates a significant reduction in elevated serum enzyme levels with extract treated animals compared to toxic control animals which can be shown in the table no 11.

Table 12: Biochemical parameters during CCl_4 induced hepatotoxicity

SNO	GROUPS	SGOT µ/l	SGPT µ/l	ALBUMIN g/dl	CREATININE g/dl	TOTAL PROTEIN mg/dl
1	Group I	90.04±3.83	75.94±1.35	5.35±0.34	0.75±0.02	6.90±0.53
2	Group II	150.3±2.28***	107.80±2.84***	6.94±0.125**	1.43±0.04***	3.16±0.32***
3	Group III	106.2±3.33***	72.94±2.13***	5.65±0.35**	0.93±0.03***	6.60±0.36***
4	Group IV	135.42±3.31*	85.83±2.54***	6.52±0.15^{ns}	1.12±0.01***	5.18±0.241***
5	Group V	128.84±2.93***	80.53±1.06***	5.58±0.17*	0.92±0.02***	6.470±0.184***

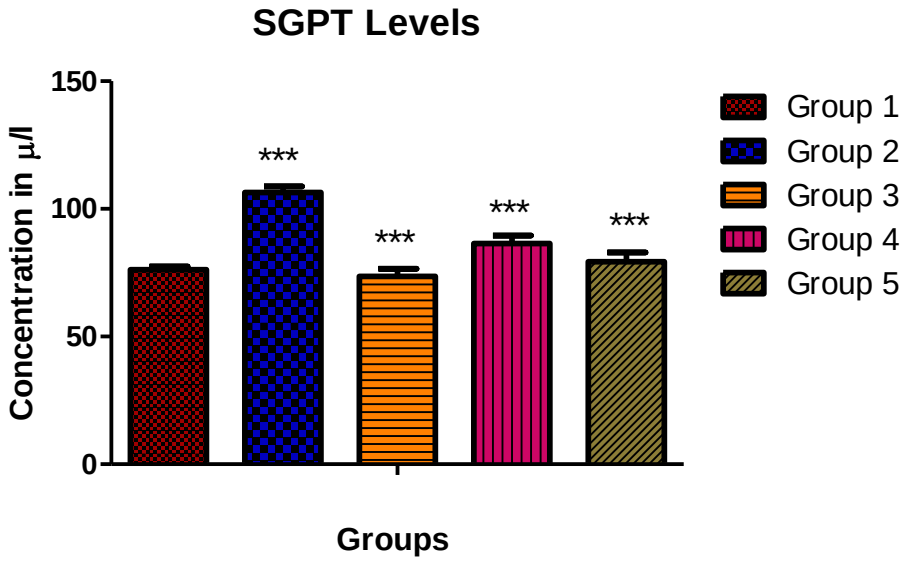
All Values are expressed as mean ± SEM, One Way Analysis of Variance, followed by Dunnett's * P<0.05, ** P<0.01 & *** P<0.001 when compared with G II; G II is compared with G I;

Figure 15: Effect of EEWC on SGOT levels in CCL4 induced hepatotoxicity in rats



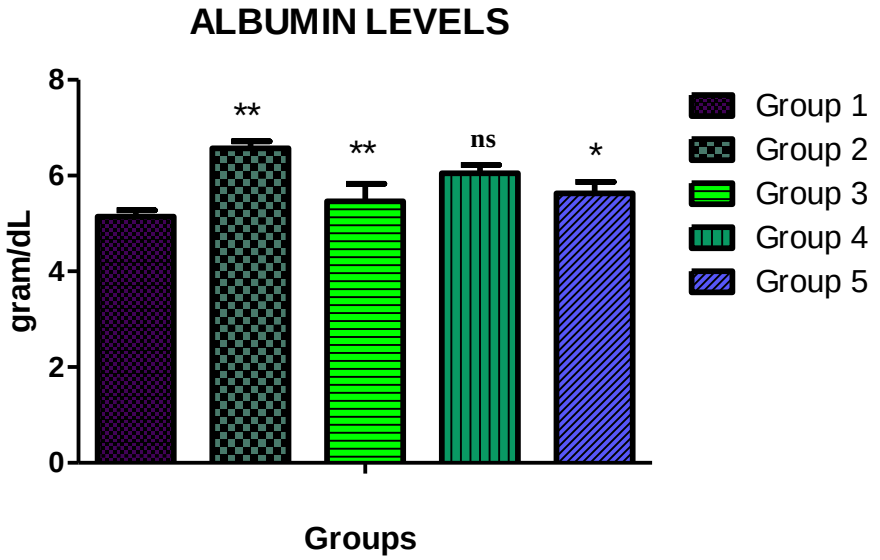
G1- Normal Group **G2** - Diseased Group; **G3 –** Standard Group; **G4** - 200 mg EEWC; **G5** - 400 mg EEWC.

Figure 16: Effect of EEWC on SGPT levels in CCL4 induced hepatotoxicity in rats



G1- Normal Group; **G2** - Diseased Group; **G3 –** Standard Group; **G4** - 200 mg EEWC; **G5** - 400 mg EEWC.

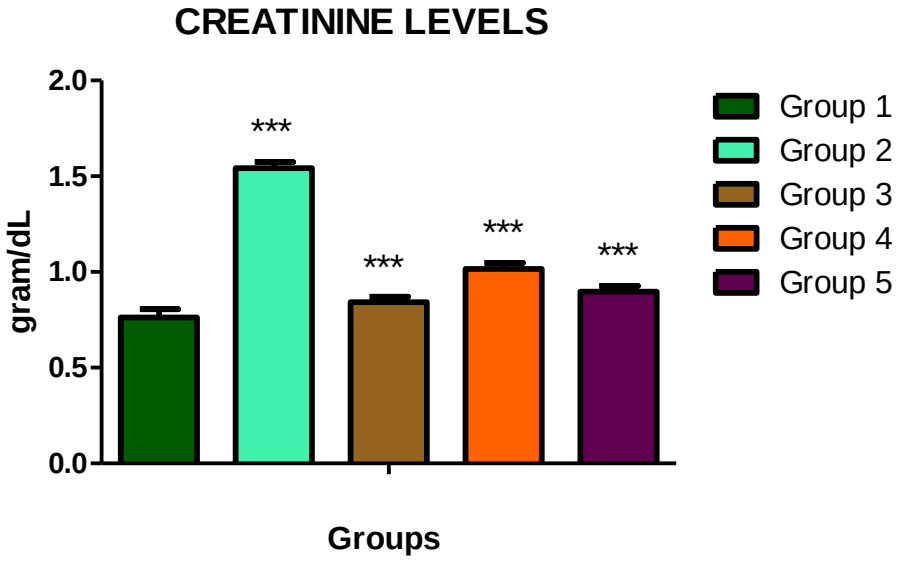
Figure 17: Effect of EEWC on Albumin levels in CCL4 induced hepatotoxicity in rats



G1- Normal Group/Control Group; **G2 -** Diseased Group;

G3 – Standard Group; **G4 -** 200 mg EEWC; **G5 -** 400 mg EEWC

Figure 18: Effect of EEWC on creatinine levels in CCL4 induced hepatotoxicity in rats



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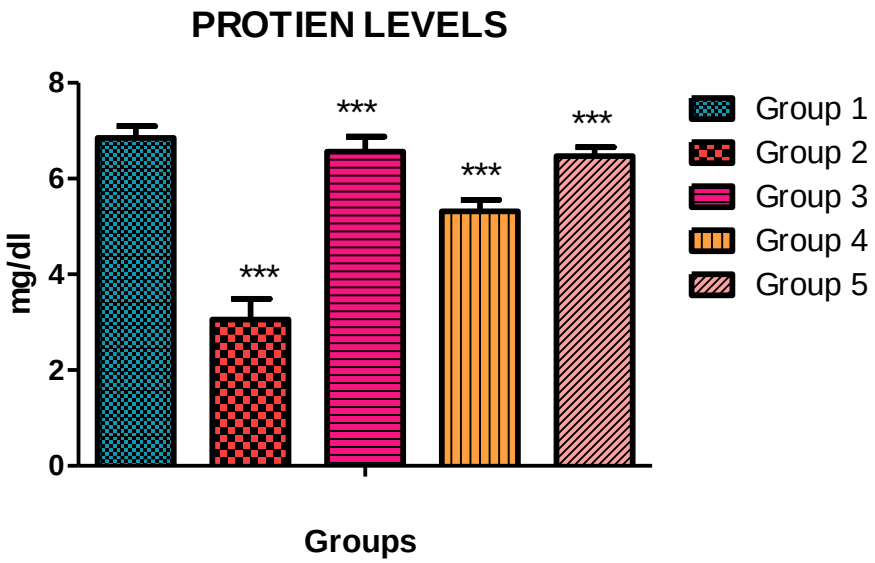
G1- Normal Group/Control Group; **G2** - Diseased Group; **G3 –** Standard Group; **G4** - 200 mg EEWC; **G5 -** 400 mg EEWC.

Figure 19: Effect of EEWC on protein levels in CCL4 induced hepatotoxicity in rats



G1- Control Group; **G2** - Diseased Group; **G3 –** Standard Group; **G4** - 200 mg EEWC; **G5 -** 400 mg EEWC.


DISCUSSION

7. DISCUSSION

Liver participates in a variety of metabolic activities perhaps by virtue of presence of number of enzymes and thus may self-expose too many toxicants, chemicals and drugs which could injure it. Traditional systems of medicine, especially Ayurveda contains number of preparations for treating liver and GIT disorders. Modern medicine provides only symptomatic relief with side effects in the treatment of liver disease.

By considering the above aspects, the present proposal of study is designed for the development and evaluation for effective management of liver diseases using *Wedelia calendulacea.*

In CCl_4 induced hepatotoxicity, CCl_4 is metabolized in endoplasmic reticulum and mitochondria with the formation of CCl_3O^-, the reactive oxidative free radical intermediate generated by cytochrome P $_{450}$, the nascent oxygen O^- resulted *via* lipoperoxidation causes rise in intracellular reactive Fe^{+2} ions, aldehyde and depletion GSH and calcium sequestration. Oxidative CCl_3O^-, also by direct covalent interaction induces degeneration of Ca^{+2} sequestrations. Failure into sequestration results in increased intercellular Ca^{+2}, aggregation by proteolytic enzymes and causes an increase in Fe^{+2} ions, which in turn by lipid peroxidation precipitates aldehyde cytotoxicity (Zimmerman MD *et al*., 1976)**.**

In the present study, coarsely powdered shade dried plant material selected for the hepatoprotective activity were subjected for extraction with ethanol. The extract after concentration is first subjected for preliminary physical and phytochemical investigation to assess the quality of plant material and understand the nature of active constituents present.

Results of phytochemical investigation revealed the presence of various phytoconstituents like glycosides, flavonoids, triterpenoids and saponin.

Phytoconstituents like flavonoids, terpenoids, saponins are known to possess hepatoprotective activities in animals (Paya M. *et al*., 1993).

After preliminary studies the extract was subjected for acute toxicity study was performed to find out LD_{50} and effective oral dose as per OECD 423 guidelines. The results showed that the LD_{50} was found to be 2000 mg/kg. Therefore, their ED_{50} is 200 mg/kg.

Hepatoprotective activity is performed against CCl_4 induced hepatotoxic rats. There were 15 groups with 6 animals in each group treated with EEWC (200 & 400 mg/kg, p. o) and silymarin (25 mg/kg, p. o) which was used as standard.

In hepatotoxicant groups, hepatotoxin gets converted into radicals in liver by action of enzymes and these attacks the unsaturated fatty acids of membranes in presence of oxygen to give lipid peroxides consequently. The functional integrity of hepatic mitochondria is altered, leading to liver damage (Tran QI. *et al*., 2001).

During hepatic damage, cellular enzymes like AST, ALT and ALP present in the liver cells leak into the serum, resulting in increased concentrations (Tran QI. *et al*., 2001). Ethanol administration for 21 days significantly increased all these serum enzymes.

Serum levels of SGPT can increase due to damage of the tissues producing acute hepatic necrosis, such as viral hepatitis and acute cholestasis. Alcoholic liver damage and cirrhosis also can associate with mild to moderate elevation of transaminases (Tran QI. *et al*., 2001).

SGOT is a mitochondrial enzyme released from heart, liver, skeletal muscle and kidney. Liver toxicity elevated the SGOT levels in serum due to the damage to the tissues producing acute necrosis, such as severe viral hepatitis and acute cholestasis. Alcoholic liver damage and cirrhosis can also associate with mild to moderate elevation of transaminases (Tran QI. *et al*., 2001).

Pretreatment with EEWC (200 & 400 mg/kg, p. o) and silymarin (25 mg/kg, p. o) exhibited an ability to counteract the hepatotoxicity by decreasing serum marker enzymes.

Serum marker enzymes such as SGPT, SGOT, total protein, albumin and creatinine showed marked increase and decrease in the level of protein. The same is observed in liver diseases in clinical practice and hence are having diagnostic importance in the assessment of liver function.

In the present study, the extract significantly reduced the elevated levels of above-mentioned serum marker enzymes and increase in the levels of protein. Hence, at this point it is concluded that the extracts possess hepato protective activity.

Finally based on improvement in serum marker enzyme levels, it is concluded that EEWC possesses hepatoprotective activity and thus supports the traditional application of the same under the light of modern science.


CONCLUSION

8. CONCLUSION

The main objective of the present study was to assess the hepatoprotective activity of ethanolic extract of *Wedelia calendulacea* leaf.

LD_{50} values of ethanolic extract of leaf of *Wedelia calendulacea* in albino rats was found safe up to the dose level of 2gm/kg confirming its non-toxic nature. The Hepatoprotective activity was studied in carbon tetra chloride induced hepatotoxic animal model. The biochemical parameters like serum SGPT, SGOT, albumin decreases and total protein increases with ethanolic extract of leaf of *Wedelia calendulacea* confirmed the hepatoprotective effect of extract under this study.

Mainly based on the improvement in serum marker enzyme levels it was concluded ethanolic extract of leaf of *Wedelia calendulacea* possesses significant hepatoprotective activity in the doses used.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