Pre-Clinical Evaluation of Therapeutic Potential of Prunetin as a Hepatoprotective Agent

Dr. Divya Singh [1*], Hina Afaq [2], Abdul Hameed [3], Yash Srivastav [4]

1 Professor (Pharmacology) Jaipur College of Pharmacy, Jaipur Rajasthan.
2 Research Scholar Jaipur College of Pharmacy, Jaipur Rajasthan.
3 Associate Professor (Pharmacology) Avadh College of Pharmacy, Lucknow, UP.
4 Assistant Professor (Pharmaceutics) Shri Venkateshwara University, Gajraula, Uttar Pradesh, India.

ABSTRACT: The term "hepatoprotective" describes a substance's capacity to shield the liver from harm. This may entail lowering inflammation, encouraging liver cell regeneration, or limiting damage from toxins. The current study's findings indicated that prunetin had phenolic content, proteins, amino acids, sterols, glycosides, saponins, flavonoids, alkaloids, and carbs. Of all the flavonoids, prunetin's hepatoprotective action is caused by glycosides and phenolic compounds. To determine the appropriate dosage of the non-toxic prunetin, additional acute toxicity research was conducted. According to an acute toxic research, the safe doses are 20 mg/kg and 40 mg/kg.
Keywords: Hepatoprotective, Prunetin, Pre-clinical Evaluation, Experiment.

I. INTRODUCTION

The liver is a most important organ with several functions. The liver has important role in metabolism and removal of drugs. A number of drugs are metabolized by the liver during phase 1 and 2 reactions which include complex processes involving cytochrome P450 enzymes. Drug metabolizing enzymes in the liver detoxify drugs and xenobiotics, ultimately leading to the acquisition of homeostasis(1). The liver is one of the most important organs in the body, performing a fundamental role in the regulation of diverse processes, among which the metabolism, secretion, storage, and detoxification of endogenous and exogenous substances are prominent (2). The second largest organ in the human body, the liver performs over 5,000 different bodily functions, such as assisting in blood coagulation, ridding the blood of toxins, converting food into nutrients, regulating hormone levels, preventing infections and illnesses, healing from injuries, and metabolizing cholesterol, glucose, and iron.

A vital center for many physiological functions is the liver. These include lipid and cholesterol homeostasis, immune system support, blood volume regulation, endocrine control of growth signaling pathways, metabolism of macronutrients, and the breakdown of xenobiotic substances, including many modern medications. Among the liver's most important roles are the processing, partitioning, and metabolism of macronutrients, which supply the energy required to power the aforementioned operations. Additionally, the liver's ability to store glucose as glycogen during feeding and reassemble glucose through the gluconeogenic route when fasting are crucial(3).

The largest network of reticulo-endothelial cells in the body, the liver plays a crucial role in the host's defense against infection. Each of the parenchymal and non-parenchymal cells that make up the organ has a distinct purpose. The biliary epithelial cells are a key target in autoimmune liver disease because they coordinate a large number of immune cells in both innate and acquired immunity. Numerous substances can cause fibrosis, necrosis, inflammation, and ultimately liver cirrhosis, fibrosis, and functional degeneration (4).

Liver has a crucial role in the directive regulation of manifold metabolic functions and physiological processes, such as the metabolism of nutrients, bile secretion and synthesis of proteins, lipids and carbohydrates as well as vitamin storage. Its ability to detoxify xenobiotics makes it particularly important in the maintenance of body health. Hepatic diseases are among leading causes of illness and mortality worldwide.Sedentary lifestyles, related to obesity and the excessive consumption of alcohol, drugs and soft drinks are shared cause of hepatic injury (5).

Liver damage can be hepatocellular, cholestatic, or mixed (includes features of both). Cholestatic damage commonly occurs due to the drug or the drug metabolite. They inhibit hepatobiliary transporter systems which are essential for bile formation and secretion of cholephilic substances and xenobiotics (6).

Hepatocellular occurs through multiple pathways including direct hepatotoxicity, and innate and adaptive immune responses. Drug-induced liver injury can be dose-dependent/intrinsic, and on most occasions, it is dose- independent/idiosyncratic. Acetaminophen usually causes drug-induced liver injury in a dose- dependent fashion. Most other drugs cause a dose-independent pattern of hepatotoxicity.

Liver metabolises a wide range of drugs the final result being to produce water soluble compounds which can be excreted in the bile. This results from phase 1 reactions mediated by cytochrome p450 including oxidation, reduction and hydrolysis reactions. This is followed by phase 2 reactions which are conjugative. After phase 1 reaction if the compound is not sufficiently polar phase 2 reaction is carried out. Sometimes phase 2 reaction is carried out first then phase 1(1).

The liver is also involved in the biochemical processes of growing, providing nutrients, supplying energy, and reproducing. In addition, it aids in the metabolism of carbohydrates and fats, in the secretion of bile, and in the storage of vitamins (3). One of the most important causes of liver dysfunction is drug-induced liver injury (DILI) which can lead to a wide spectrum of symptoms ranging from mild non-specific symptoms like asymptomatic transaminitis, acute hepatitis, chronic hepatitis, cholestasis to liver failure. It can be caused by a multitude of prescription drugs, herbal and dietary supplements and commonly leads to withdrawal of the drug from the market.

The functional unit of the liver is the lobule. Each lobule is hexagonal, and a portal triad (portal vein, hepatic artery, bile duct) sits at each corner of the hexagon. The foundation of the lobule is composed of hepatocytes, which have physiologically distinct apical and basolateral membranes. Based on function and perfusion, hepatocytes are divided into 3 zones (7).

1. Zone I is considered to be the periportal region of hepatocytes and is the best perfused and first to regenerate due to its proximity to oxygenated blood and nutrients. Due to its high perfusion, zone I plays a large role in oxidative metabolisms such as beta-oxidation, gluconeogenesis, bile formation, cholesterol formation, and amino acid catabolism.
2. Zone II is defined as the pericentral region of the hepatocytes, and zone II sits between zones I and III.
3. Zone III has the lowest perfusion due to its distance from the portal triad. It plays the

largest role in detoxification, biotransformation of drugs, ketogenesis, glycolysis, lipogenesis, glycogen synthesis, and glutamine formation.

There are many classes of drugs causing drug-induced liver injury including nonsteroidal anti- inflammatory drugs (NSAIDs), anti-infective drugs (anti-tubercular drugs), anti-cancer drugs, hormonal drugs, immunosuppressive agents, sedative, and neuropsychiatric drugs (8). The most common drug implicated in drug-induced liver injury is acetaminophen. Antibiotics are the class of drugs most commonly causing liver toxicity, and amoxicillin-clavulanate stands out as the most common drug in this class. Additionally, herbal supplements cause a variety of symptoms, but their usage remains under-reported.

1.1 Classification

A number of unpleasant reactions brought on by different pharmaceutical medications (prescription and over-the-counter), herbal remedies, and nutritional supplements through a variety of pathways are together referred to as drug-induced liver injury (DILI) . With estimated yearly incidence rates ranging from 1.3 to 19.1 per 100,000 people worldwide, as well as regional variations in prevalence and etiology,

DILI is a serious global health concern. Whereas liver damage from a drug overdose manifests within hours to days after drug exposure, DILI usually happens days to months later. Hepatotoxicity or DILI symptoms differ from person to person. Acute liver failure develops as a result of repeated bouts of acute liver injury, but in certain mild or chronic cases, individuals may not exhibit any symptoms of liver damage (9).

The clinical symptoms, signs and patterns of liver test abnormalities of drug induced liver injury can mimic virtually any form of liver disease – from acute viral hepatitis to gall stone disease with biliary obstruction, acute fatty liver and even chronic hepatitis and cirrhosis. There are no specific findings or laboratory tests that definitely prove that a suspected drug, nutritional supplement or herbal product is the cause of the injury.

Drug induced liver injury is a diagnosis of exclusion and relies upon clinical judgment and knowledge about the potential of the agent to cause liver injury versus the likelihood that other forms of liver diseases are the cause. Various researches elaborated that among the proposed factors for liver injury are insulin resistance, inflammation, oxidative stress, and bile acid toxicity. However, new conceptual approaches focus more on the

adipose tissue (AT) and its modulating lipotoxic property on liver function. In more detail, the release of fatty acids from dysfunctional and insulin-resistant adipocytes results in hepatic lipotoxicity. This is caused by the accumulation of triglyceride-derived toxic metabolites and the subsequent beginning of inflammatory pathways, cellular disfunction, and lipoapoptosis (10)

In judging the likelihood of drug induced liver disease, six features are important:
- The onset after starting the drug: time to onset [latency]
- The recovery after stopping the drug: time to recovery [challenge]
- The clinical pattern: injury pattern and clinical phenotype [phenotype]
- Exclusion of other causes of liver injury [differential diagnosis]
- Whether the drug is a known cause of liver injury [likelihood]
- Response to reexposure, whether inadvertent or intentional [rechallenge]

Drug induced liver disease can be classified into three types of liver damage (11)
1. Hepatocellular,
2. Cholestatic,
3. Mixed hepatocellular-cholestatic.

These designations refer to histologic features of injury, but are usually defined based upon the pattern of serum enzyme elevations.
1. **Hepatocellular injury**: A noticeable pattern of harm to the hepatocellular structure characterizes drug-induced liver disease, which is similar to acute viral hepatitis. If a liver biopsy is available, it typically reveals mild bile stasis and significant liver cell necrosis and inflammation, at least in the early stages.
a. Acute hepatocellular injury: severe inflammation with necrosis and apoptosis seen in isoniazid (INH), aspirin, and phenytoin
b. Chronic hepatocellular injury: findings as above with fibrosis resembling other chronic liver disease seen in amoxicillin-clavulanate, valproic acid, amiodarone

2. **Cholestatic injury**: A cholestatic image of drug-induced liver damage is similar to choledocholithiasis or bile duct blockage. The results of a liver biopsy typically show bile stasis, portal inflammation, and either bile duct and ductule damage or proliferation.

Clinically, jaundice and itching are the most common signs.
a. Acute cholestasis: bile plugging with hepatocellular cholestasis, commonly seen in anabolic steroid use
b. Chronic cholestasis: bile stasis, portal inflammation, bile jury, bile plugs, and duct paucity seen in amoxicillin-clavulanate

3. **Mixed hepatocellular-cholestatic injury.** Many medicines cause a combination of hepatocellular and cholestatic injury, which is actually the most prevalent pattern of drug-induced liver injury and only rarely seen in other acute liver disease forms. Liver biopsy results in significant hepatocyte necrosis and inflammation along with noticeable bile stasis in cases of mixed damage.
a. Steatosis: Micro vesicular often related to mitochondrial injury seen in tetracycline and valproic acid
b. Zonal necrosis: Usually in intrinsic drug induced hepatotoxicity and associated with poor outcomes, seen in acetaminophen toxicity
c. Granulomas: Associated with milder injury and can result from many drugs or even talc exposure through the bloodstream

Acute and chronic liver disorders (CLDs) are becoming more common worldwide, affecting people's quality of life and potentially leading to life-threatening complications. Antivirals have reduced the burden of advanced liver disease caused by hepatitis B, but it will be challenging to eradicate it in the near future. Although the burden of hepatitis C has decreased due to very effective directly acting antiviral medication, this has been somewhat countered by an increase in IV drug misuse. Aside from managing risk factors, there is no medication to treat the pandemic of non-alcoholic fatty liver disease or the recent, concerning rise in alcohol-related liver disease (12).

In patients with cirrhosis, acute-on-chronic liver failure (ACLF) is a syndrome marked by acute decompensation and the onset of organ failure or failures that are linked to a high death rate.1 The current care of ACLF is centered on organ support, and there is currently no viable strategy to stop its growth.2. Lack of understanding of the molecular pathophysiology of cirrhosis progression and ACLF development is the primary cause of the lack of a viable treatment (13).

Chronic liver disease (CLD) has become a leading cause of death worldwide and poses a

serious threat to public health. Although there are a number of reasons why CLDs occur, With about 50% of cases, non-alcoholic fatty liver disease (NAFLD) is the main cause of these. A variety of metabolic disorders, including obesity, diabetes, dyslipidemia, hypertension, and insulin resistance, are frequently linked to non-alcoholic fatty liver disease (NAFLD), which is defined by the intracellular accumulation of lipids in hepatocytes. From a simple fatty infiltration to progressive non-alcoholic steatohepatitis (NASH), a more severe disorder involving inflammation and further hepatocyte destruction, NAFLD constitutes a distinct spectrum of liver disease (14).

Nonalcoholic fatty liver disease (NAFLD) has been renamed metabolic-associated fatty liver disease (MAFLD). It is a common positive diagnosis that is based on metabolic parameters and is not influenced by alcohol consumption. The current diagnostic criteria for MAFLD include the presence of at least two metabolic risk factors, such as high waist circumference, hypertension, hypertriglyceridemia, hypo-HDL cholesterolemia, prediabetes, insulin resistance, and elevated high-sensitivity C-reactive protein levels, as well as evidence of hepatic steatosis (as shown by biopsy, imaging, or validated serum biomarkers) and one of the following criteria: overweight/obesity, type 2 diabetes mellitus, or metabolic dysregulation (15).

Alcohol misuse, non-alcoholic steatohepatitis (NASH), viral hepatitis, and autoimmunity are the main causes of chronic liver disease, which can lead to cirrhosis. Serious health issues, hospital stays, and higher mortality rates are frequently linked to liver illness.One Clinical management is crucial for the physical symptoms and course of the disease, but patients' experiences with the disease and quality of life are typically overlooked, despite the fact that these factors are crucial for determining a patient's overall health and for organizing liver care.2. The ignorance of the emotional and social symptoms of liver patients is a significant obstacle. These are frequently ignored and left unsaid.3. This is an undesirable circumstance since liver disease frequently has a detrimental effect on a patient's social and familial life, career, and financial standing (16).

1.2 Pathophysiology
The pathogenesis of Drug induced liver inflammation (DILI) or drug induced hepatotoxicity can be divided into 2 mechanisms: Intrinsic and Idiosyncratic.

The intrinsic mechanism is both predictable and reproducible from drugs that are known to cause liver injury in a dose-dependent manner with a short latency period (10, 17, 18).

Idiosyncratic drug-induced liver injury (iDILI) is a rare adverse drug reaction that occasionally leads to acute liver failure or even death. An aging population that uses more drugs, a constant influx of newly developed drugs, and a growing risk from herbal and dietary supplements of uncertain quality can lead to an increase in iDILI. Although the exact mechanism is unknown, it is thought to be due to a combination of host, drug, and environmental factors. The host factors are patient age, gender, genetic polymorphisms, immune status, and metabolism. Drug factors include the dose, duration, weight, and degree of lipophilicity. Environmental factors consist of concomitant alcohol use, diet, tobacco, and toxins (18-20). Idiosyncratic DILI mechanisms can be divided into immune-mediated (allergic) liver injury from hypersensitivity or non-immune-mediated metabolic (non- allergic) mechanisms from mitochondrial injury (17).

Fibrosis is the prelude to liver cirrhosis, which is the pathological end consequence of several chronic liver disorders. Liver fibrosis and cirrhosis are caused and progressed by a variety of cell types, cytokines, and miRNAs. One of the key processes in fibrosis is the activation of hepatic stellate cells (HSCs). One of the main causes of hepatic dysfunction in liver cirrhosis is the defenestration and capillarization of liver sinusoidal endothelial cells. Hepatocytes are destroyed by activated Kupffer cells, which also promote HSC activity (21).

The principal treatment for drug-induced hepatotoxicity is the removal of the offending agent. N-acetyl-cysteine (NAC) is the treatment for intrinsic DILI secondary to acetaminophen toxicity, as this promotes the regeneration of glutathione, leading to the detoxification of the toxic metabolite (6, 17). The other specific therapy that is available is L-carnitine for valproic acid overdose.

1.3 Sign and Symptoms
Liver damage has various potential causes, as the liver starts to damage, various sign and symptoms persist.

At first, these conditions may not cause any symptoms. As the damage worsens, many symptoms appear like loss of appetite, fatigue, nausea, discomfort, mild pain, unexplained weight loss and vomiting.

Cirrhosis symptoms related to declining liver function include:

- Jaundice (yellow tint to your skin and eyes).
- Pruritus (itchy skin, but with no visible rash).
- Dark-colored pee and light-colored poop.
- Digestive difficulties, especially with fats.
- Small yellow bumps of fat deposits on your skin or eyelids.
- Unexplained weight loss and muscle loss.
- Hepatic encephalopathy (confusion, disorientation, mood changes).
- Motor dysfunction (twitching, tremors or lapses in muscle control).

- Disruptions to your menstrual cycle.
- Enlarged male breast tissue and shrunken testes in people AMAB.

1.4 Management of liver disease
- Herbals
- Deoxycholic acid
- Wheat grass
- Healthy diet
- Less oily food
- Healthy life style
- Vitamin C rich food

Figure 1: Management of liver disease

Lose Weight if Overweight or Obese

Control Blood Sugar Levels

Manage Other Health Conditions

Lower Cholesterol and Triglycerides

Eat a Healthy, Balanced Diet

Take Vitamins and Supplements Wisely

2.1 Basis of the study

Flavonoids are polyphenolic chemicals that are prevalent in fruits and flowers and found in nature. Phytochemicals belonging to the flavonoid class have drawn attention from scientists due to their significant pharmacological properties. Numerous studies have demonstrated the antibacterial, antioxidative, antifungal, analgesic, antiviral, anti-inflammatory, antitumor, antiparasitic, and antiallergic properties of phytochemicals belonging to the flavonoid class. Prunetin is an O-methylated isoflavone that is found in licorice, red cherries, soybeans, and legumes. It is a member of the phytochemical phytoestrogen class.

There are very less hepatoprotective agent available now a days. Liver is the most important organ in the body. If liver will get damaged by any means, protection of liver is very important. Prunetin having antioxidant property and anti-

inflammatory property may prove a better therapeutic potential as hepatoprotective agent. This study is for pre-clinical evaluation of therapeutic potential of prunetin as a hepatoprotective agent.

2.2 Need of the study

This type of study is very much important now a days to search an important agent as hepatoprotective. Liver is the most vital organ in the body. Due to obesity and many reasons liver got affected. It needs to be protected. There are very few drugs available till now for hepatoprotection. In the field of hepatoprotection, very few works have been carried out till now. Liver is the vital organ of the body so it is very important to take care of it. Plant extracts have been extensively used for the liver protective action. This study is only for preclinical evaluation

of Prunetin as hepatoprotective agent and extensive clinical trials needs to be done in this particular research.

Drug Identification.

The most common reasons for using herbal therapy for treatment are dissatisfaction with conventional medicine, previous favourable experiences, positive aspects of herbal medicine, and family traditions. Flavonoids are polyphenolic compounds found to be present in nature and abundant in flowers and fruits. Flavonoid class phytochemicals have gained interest in the scientific field because of their important pharmacological activities (22). Several scientific studies have revealed anti-bacterial, anti-oxidant, anti-fungal, analgesic, anti-viral, anti-inflammatory, anti-tumor, anti-parasitic and anti-allergic activities of flavonoid class phytochemicals. Prunetin is an O-methylated isoflavone that belongs to the phytochemical phytoestrogen class, found to be present in licorice, red cherry, soybean and legumes.

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Hepatoprotective effects of natural compounds have been frequently attributed to their antioxidant properties and the ability to mobilize endogenous antioxidant defense system. Because of involvement of oxidative stress in virtually all mechanisms of liver injury, it is a reasonable presumption that antioxidant properties of these compounds may play a key role in the mechanism of their hepatoprotective activity. Nevertheless, growing evidence suggests that other pharmacological activities of natural compounds distinct from antioxidant are responsible for their therapeutic effects (22).

Polyphenols are naturally occurring compounds found largely in the fruits, vegetables, cereals and beverages. Fruits like grapes, apple, pear, cherries and berries contains up to 200–300 mg polyphenols per 100 grams fresh weight. The products manufactured from these fruits, also contain polyphenols in significant amounts. Typically, a glass of red wine or a cup of tea or coffee contains about 100 mg polyphenols. Cereals, dry legumes and chocolate also contribute to the polyphenolic intake (23, 24).

Polyphenol compounds have been shown to provide health benefits in preventing various diseases, including cancer, metabolic syndrome, diabetes, non-alcoholic liver disease, and periodontal disease. Traditionally, the health benefits of polyphenolic compounds have been attributed to their antioxidant activity(25). Knowledge of chemical plant components is essential for quality control analysis of plants, extracts, or any formulation containing chemical plant components. Existing compounds or groups of compounds can serve as "biological markers." The presence and concentration of the biologically active ingredients will be used to decide the drug's authenticity or formulation.

Prunetin, a flavonoidal compound derived from Glycyrrhizae Radix, a medicinal plant used for controlling various inflammatory diseases in folk medicine, showed the biological activities including anti-inflammatory effect (26). Prunetin showed anti-obesity potential and inhibited human liver aldehyde dehydrogenase (27, 28)

Prunetin characteristics
1. Molecular Formula- C16H12O5
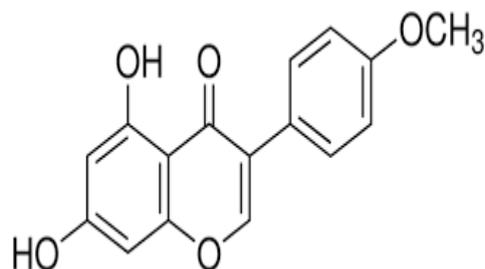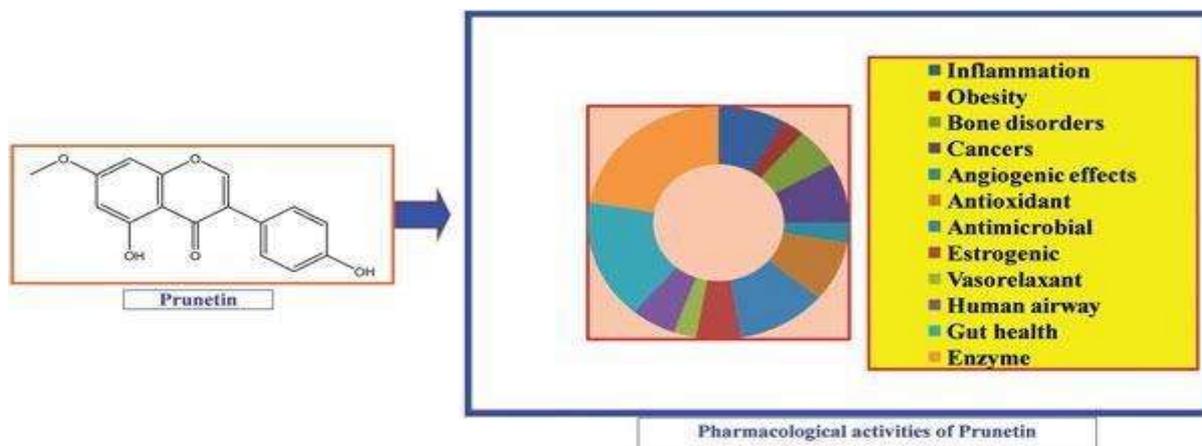2. Chemical Structure

Figure 1: Structure of 5-hydroxy-3-(4-hydroxyphenyl)-7-methoxychromen-4-one

3. Synonyms- Prunetin 552-59-0, Prunusetin,5-hydroxy-3-(4-hydroxyphenyl)-7-methoxy-4H-chromen-4-one, 5,4'-dihydroxy-7-methoxyisoflavone
4. Molecular Weight - 284.26 g/mol
5. Boiling Point- 546.50 °C.
6. Melting Point- 246 - 248 °C
7. Solubility-Soluble in Chloroform,Dichloromethane,Ethyl Acetate,DMSO,Acetone,etc.
8. Therapeutic properties

Therapeutic properties of prunetin in medicine.
Figure 2: Therapeutic Properties of Prunetin

9. Pharmacokinetics

GI absorption	High
BBB permeant	No
P-gp substrate	No
CYP1A2 inhibitor	Yes
CYP2C19 inhibitor	No
CYP2C9 inhibitor	No
CYP2D6 inhibitor	Yes
CYP3A4 inhibitor	Yes
Log Kp(skin permeation) -	5.91 cm/s

10. Pharmacological Action:

S. No.	Pharmacological Properties
1	Anti-allergy
2	Anti-Asthmatic Activity
3	Anti-inflammatory
4	Antidepressant
5	Anti-oxidant
6	Cardiovascular activity
7	Anti-Spasmodic Activity

Justification of study

Plant extracts have been widely used for the hepatoprotective action. Among the various extracts, flavonoids have important hepatoprotective action. Prunetin has flavanoidal activity, antioxidant activity. In this regard, this study is aimed to check the hepatoprotective action of flavonoids.

II. MATERIAL AND METHODS

Preliminary Phytochemical evaluation

Preliminary phytochemical examinations of Prunetin was carried out as per the Standard Methods.[76, 77]

Detection of alkaloids:

Extracts were dissolved individually in dilute Hydrochloric acid and filtered. The filtrates were used to test for the presence of alkaloids.

a. **Mayer's Test:** 2-3 ml of Filtrate was treated with Mayer's reagent (Potassium Mercuric iodide). Formation of a yellow cream precipitate indicates the presence of Alkaloids.

b. **Wagner's test:** Filtrates were treated with Wagner's reagent (Iodine in potassium iodide). Formation of brown/reddish brown precipitate indicates the presence of alkaloids.

c. **Dragendorff's test:** Filtrates were treated with Dragendroff's reagent (solution of potassium bismuth iodide). Formation of red precipitate indicates the presence of alkaloids.

d. **Hager's test:** 2-3 ml of Filtrate was treated with Hager's reagent (saturated picric acid solution). Formation of yellow colored precipitate indicates the presence of alkaloids.

Detection of carbohydrates:

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

a. **Molisch's Test:** 2-3 ml of Filtrate was treated with 2 drops of alcoholic α-naphthol solution in a test tube and 2 ml of Conc. Sulphuric acid was added carefully along the sides of the test tube. Formation of violet ring at the junction indicates the presence of Carbohydrates.

b. **Tests for reducing sugars**:

i. **Benedict's test:** Filtrates were treated with Benedict's reagent and heated on water bath. Formation of orange red precipitate indicates the presence of reducing sugars.

ii. **Fehling's test:** Filtrates were hydrolyzed with dil. HCl, neutralized with alkali and heated with Fehlings A & B solutions. Formation of red precipitate indicates the presence of reducing sugars.

c. **Test for monosaccharide**

i. **Barfoed's test:** mix equal volume of barfoed's reagent and the filtrate. Heat the mixture for 1-2 min. in water bath, appearance of red precipitate indicates monosaccharide.

Detection of glycosides:

Extracts were hydrolyzed with dil. HCl, and then subjected to test for glycosides.

a. **Modified Borntrager's Test (for anthraqinone glycosides):** Extracts were treated with Ferric Chloride solution and immersed in boiling water for about 5 minutes. The mixture was cooled and shaken with an equal volume of benzene or chloroform. The benzene layer was separated and treated with ammonia solution. Formation of rose- pink colour in the ammoniacal layer indicates the presence of anthranol glycosides.

b. **Legal's test (for cardenolides):** Extracts were treated with sodium nitroprusside in pyridine and methanolic alkali. Formation of pink to blood red colour indicates the presence of cardiac glycosides.

c. **Keller – killiani test (for deoxysugars):** Extracts were treated with few drops of glacial acetic acid and added 1 drop of 5% ferric chloride and from the sides of test tube added few drops of conc. Sulphuric acid. Appearance of reddish brown color of the junction and bluish green upper layer indicates presence of deoxysugars.

Detection of saponins

a. **Froth Test:** Extracts were diluted with distilled water to 20ml and this was shaken in a graduated cylinder for 15 minutes. Formation of 1 cm layer of foam indicates the presence of saponins.

b. **Foam test:** Small amount of extract was shaken with little quantity of water. If foam produced persists for ten minutes it indicates the presence of saponins.

Detection of phytosterols

a. **Salkowski's Test:** Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of Conc. Sulphuric acid, shaken and allowed to stand. Appearance of golden yellow color indicates the presence of triterpenes.

a. **Liebermann Burchard's test:** Extracts were treated with chloroform and filtered. The filtrates were treated with few drops of acetic anhydride, boiled and cooled. Conc. Sulphuric acid was added carefully along the sides of the test tube. Formation of brown ring at the junction indicates the presence of phytosterols.

b. **Tshugajeu test:** Extracts were treated with chloroform and filtered. Excess of acetyl chloride and a pinch of Zinc Chloride was added, kept aside for some time till the reaction was complete and then warmed on waterbath. Appearance of eosin red color indicates the presence of triterpenes.

Detection of fixed oils & fats

a. **Stain Test:** Small quantities of extracts were pressed between two filter papers. An oily stain on filter paper indicates the presence of fixed oil.

Detection of resins

a. **Acetone-water Test:** Extracts were treated with acetone. Small amount of water was added and shaken. Appearance of turbidity indicates the presence of resins.

Detection of phenols.

a. **Ferric Chloride Test:** Extracts were treated with few drops of ferric chloride solution. Formation of bluish black color indicates the presence of phenols.

Detection of tannins

a. **Gelatin Test:** To the extract, 1% gelatin solution containing sodium chloride was added. Formation of white precipitate indicates the presence of tannins.

Detection of flavonoids

a. **Alkaline Reagent Test:** Extracts were treated with few drops of sodium hydroxide solution. Formation of intense yellow color, which becomes colorless on addition of dilute acid, indicates the presence of flavonoids.

b. **Lead acetate Test:** Extracts were treated with few drops of lead acetate solution. Formation of yellow color precipitate indicates the presence of flavonoids.

c. **Shinoda Test:** To the alcoholic solution of extracts, a few fragments of magnesium ribbon and Conc.HCl was added. Appearance of magenta color after few minutes indicates presence of flavonoids.

d. **Zinc hydrochloric acid reduction Test:** To the alcoholic solution of extracts, a pinch of Zinc dust and Conc. HCl was added. Appearance of magenta color after few minutes indicates presence of flavonoids.

Detection of proteins and aminoacids

a. **Xanthoproteic Test:** The extracts were treated with few drops of concentrated Nitric acid solution. Formation of yellow color indicates the presence of proteins.

b. **Ninhydrin test:** To the extract, 0.25% ninhydrin reagent was added and boiled for few minutes. Formation of blue color indicates the presence of amino acid.

c. **Biuret Test:** The extracts were treated with 1 ml of 10% sodium hydroxide solution and heated. To this a drop Of 0.7% copper sulphate solution was added. Formation of purplish violet color indicates the presence of proteins.

Detection of diterpenes

a. **Copper acetate Test:** Extracts were dissolved in water and treated with few drops of copper acetate solution. Formation of emerald green colour indicates the presence of diterpenes.

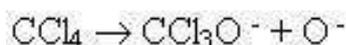## 2.2 IN-VIVO HEPATOPROTECTIVE SCREENING OF PRUNETIN

2.2.1 Hepatotoxins and mode of liver damage

Hepatoprotective agents are those compounds, which mitigate the liver injury caused by hepatotoxic agents.[1] Hepatoprotective effects of plant drugs and herbal formulations are studied against chemicals(alcohol, CCl_4, alcohol-CCl_4, beta galactosamine, thioacetamide) and drugs(paracetamol, nimesulide, antitubercular drugs like isoniazid, rifampicin etc.) induced hepatotoxicity in rats and mice as they virtually mimic any form of naturally-occurring liver disease.

Hepatotoxicity from drugs and chemicals is the commonest form of iatrogenic disease. Some of the inorganic compounds producing hepatotoxicity are arsenic, phosphorus, copper and iron. The organic agents include certain naturally-occurring plant toxins such as pyrrolizidine alkaloids, mycotoxins and bacterial toxins. The synthetic group of organic compounds is a large number of medicinal agents. In addition, exposure to hepatotoxic compounds may be occupational, environmental or domestic that could be accidental, homicidal or suicidal ingestion. the paper discusses various models used for screening hepatoprotective drugs.

CCl₄ induced hepatotoxicity

Liver injury due to carbontetrachloride in rats was first reported in1936[3] and has been widely and successfully used by many investigators. [4,5]Carbontetrachloride is metabolized by cytochrome P-450 in endoplasmic reticulum and mitochondria with the formation of $CCl_3\ O^-$, a reactive oxidative free radical, which initiates lipid peroxidation.

$$CCl_4 \rightarrow CCl_3O^- + O^-$$

Administration of a single dose of CCl_4 to a rat produces, within 24 hrs, a centrilobular necrosis and fatty changes. The poison reaches its maximum concentration in the liver within 3 hrs of administration. Thereafter, the level falls and by 24 hrs there is no CCl_4 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.

Galactosamine induced hepatotoxicity

D-Galactosamine induced liver damage has been extensively used as an experimental model. Galactosamine produces diffuse type of liver injury simulating viral hepatitis. It presumably disrupts the synthesis of essential uridylate nucleotides resulting in organelle injury and ultimately cell death. Depletion of those nucleotides would impede the normal synthesis of RNA and consequently would produce a decline in protein synthesis. This mechanism of toxicity brings about an increase in cell membrane permeability leading to enzyme leakage and eventually cell death. The cholestasis caused by galactosamine may be from its damaging effects on bile ducts or ductules or canalicular membrane of hepatocytes Galactosamine decrease the bile flow and its content i.e. bile salts, cholic acid and deoxycholic acid. Galactosamine reduces the number of viable hepatocytes as well as rate of oxygen consumption. Dose of D-Galactosamine: 400 mg/kg, I.P.

Thioacetamide induced hepatotoxicity

Thioacetamide interferes with the movement of RNA from the nucleus to cytoplasm which may cause membrane injury. A metabolite of thioacetamide (perhaps s-oxide) is responsible for hepatic injury. Thioacetamide reduce the number of viable hepatocytes as well as rate of oxygen consumption. It also decreases the volume of bile and its content i.e. bile salts, cholic acid and deoxycholic acid. Dose of thioacetamide: 100 mg/kg, S.C.

Alcohol induced hepatotoxicity

Liver is among the organs most susceptible to the toxic effects of ethanol. Alcohol consumption is known to cause fatty infiltration, hepatitis and cirrhosis. Fat infiltration is a reversible phenomenon that occurs when alcohol replaces fatty acids in the mitochondria. Hepatitis and cirrhosis may occur because of enhanced lipid peroxidative reaction during the microsomal metabolism of ethanol. It is generally accepted that alcohol can induce in vivo changes in membrane lipid composition and fluidity, which may eventually affect cellular functions. Among the mechanisms responsible for effects of alcohol, an increase in hepatic lipid peroxidation leads to alteration in membrane phospholipid composition.

The effects of ethanol have been suggested to be a result of the enhanced generation of oxyfree radicals during its oxidation in liver. The peroxidation of membrane lipids results in loss of membrane structure and integrity. These results in elevated levels of ¡-glutamyl transpeptidase, a membrane bound enzyme in serum. Ethanol inhibits glutathione peroxidase, decrease the activity of catalase, superoxide dismutase, along with increase in levels of glutathione in liver. The decrease in activity of antioxidant enzymes superoxide dismutase, glutathione peroxidase are speculated to be due to the damaging effects of free radicals produced following ethanol exposure or alternatively could be due to a direct effect of acetaldehyde, formed by oxidation of ethanol. [10]Alcohol pre-treatment stimulates the toxicity of CCl_4 due to increased production of toxic reactive metabolites of CCl_4, namely trichloro-methyl radical by the microsomal mixed function oxidative system. This activated radical binds covalently to the macromolecules and induces peoxidative degradation of membrane lipids of endoplasmic reticulum rich in polyunsaturated fatty acids. This lipid peroxidative degradation of biomembranes is the principle cause of hepatotoxity.

Paracetamol induced hepatotoxicity

Paracetamol, a widely used analgesic and antipyretic drug, produces acute liver damage in high doses. Paracetamol administration causes necrosis of the centrilobular hepatocytes characterized by nuclear pyknosis and eosinophilic cytoplasm followed by large excessive hepatic lesion. The covalent binding of N-acetyl-P-benzoquinoneimine, an oxidative product of

paracetamol to sulphydryl groups of protein, result in lipid peroxidative degradation of glutathione level and thereby, produces cell necrosis in the liver. Dose of Paracetamol: 1 gm/kg P.O.

Antitubercular drugs induced hepatotoxicity

Drug induced hepatotoxicity is a potentially serious adverse effect of the currently used antitubercular therapeutic regimens containing Isoniazid (INH), Rifampicin and Pyrazinamide. Adverse effects of antitubercular therapy are sometimes potentiated by multiple drug regimens. Thus, though INH, Rifampicin and Pyrazinamide each in itself are potentially hepatotoxic, when given in combination, their toxic effect is enhanced. INH is metabolized to monoacetyl hydrazine, which is further metabolized to a toxic product by cytochrome P$_{450}$ leading to hepatotoxicity. Patients on concurrent rifampicin therapy have an increased incidence of hepatitis. This has been postulated due to rifampicin-induced cytochrome P$_{450}$ enzyme-induction, causing an increased production of the toxic metabolites from acetyl hydrazine (AcHz). Rifampicin also increases the metabolism of INH to isonicotinic acid and hydrazine, both of which are hepatotoxic. The plasma half life of AcHz (metabolite of INH) is shortened by rifampicin and AcHz is quickly converted to its active metabolites by increasing the oxidative elimination rate of AcHz, which is related to the higher incidence of liver necrosis caused by INH and rifampicin in combination. Rifampicin induces hydrolysis pathway of INH metabolism into the hepatotoxic metabolite hydrazine. Pharmacokinetic interactions exist between rifampicin and pyrazinamide in tuberculosis patients, when these drugs are administered concomitantly. Pyrazinamide decrease the blood level of rifampicin by decreasing its bioavailability and increasing its clearance. Pyrazinamide, in combination with INH and rifampicin, appears to be associated with an increased incidence of hepatotoxicity.[12]

Models to Evaluate Effect of Drugs on Liver

As liver is a multifunctional organ, a battery of liver function tests is employed to evaluate the effect of drug on liver, which are
- Non-invasive functional methods: -
a. Ascorbic acid content in urine
b. Pentobarbitone induced sleeping time
c. Bromosulphthaline clearance test
- Biochemical analysis of blood for:-
1. SGPT

2. SGOT
3. Alkaline phosphatase
4. Serum bilirubin
5. Total proteins
- Morphological test-Wet weight of liver/100 gm body weight
- Biochemical analysis of liver homogenate to detect:-

a. Free radical scavengers
b. Glutathione
c. Lipid peroxidation
d. Superoxide dismutase
e. Catalase
f. Glutathione peroxidase and
g. Lipid profile
h. Total protein

- Histopathology of liver

Ascorbic acid content in urine

Measurement of ascorbic acid content of urine is reported as a non-invasive test for screening hepatoprotective agents against CCl_4 induced hepatotoxicity in rats. CCl_4, a pharmacological tool to produce liver damage, reduces the excretion of ascorbic acid in rats.

Hexobarbitone or zoxazolamine induced sleeping time

Toxic liver prolongs duration of sleeping time for pentobarbitone, hexobarbitone, zoxazolamine etc in mice, rats.
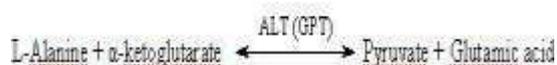
Bromosulphthaline clearance test

The liver normally clears bromosulphthalein (BSP), a dye, from the blood. The level of BSP in the blood after intravenous injection of BSP is a sensitive guide to hepatic damage. During the passage of BSP from the plasma to the bile, it undergoes storage, metabolism and excretion by the liver. The abnormal functional effects produced by CCl_4 leads to the retention of BSP in blood.

Serum and hepatocyte enzyme

AST i.e. Aspartate Transaminase (SGOT), and ALT i.e. Alanine Transaminase (SGPT), are both sensitive markers of hepatocellular injury. When the liver cell is injured or dies, these proteins can leak through the liver cell membrane into the circulation and serum levels will rise. ALT or SGPT is a cytosolic enzyme primarily present in the liver. Its normal serum level is 10-35 Karmel

units/ml. ALT reversibly catalyses amino group from alanine to α-ketoglutarate.

$$L\text{-Alanine} + \alpha\text{-ketoglutarate} \xrightleftharpoons{ALT\,(GPT)} Pyruvate + Glutamic\ acid$$

ALT levels are very high in patients of viral hepatitis and hepatic necrosis, 10 to 200 fold higher in patients of post hepatic jaundice, intrahepatic cholestasis and below 10 fold in patients of metastatic carcinoma, cirrhosis and alcoholic hepatitis.
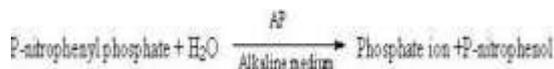
AST or SGOT is a mitochondrial enzyme released from heart, liver, skeletal muscles and kidney. Its normal serum level is 10-40 Karmel units/ml AST reversibly catalyses transfer of amino group from aspartate to α-ketoglutarate.

$$L\text{-Aspartate} + L\text{-}\alpha\text{-oxaglutarate} \xrightleftharpoons{AST\,(GOT)} L\text{-oxaloacetate} + L\text{-glutamate}$$

AST levels are 10 to 200-fold elevated in patients with acute hepatic necrosis, viral hepatitis, CCl_4 and drug induced poisoning.

Alkaline phosphatase
Serum alkaline phosphatase is produced by many tissues, especially bone, liver, intestine and placenta and is excreted in the bile. In the absence of bone disease and pregnancy, an elevated serum alkaline phosphatase levels generally reflect hepatobiliary disease. The mechanism of elevated ALP levels may be due to defective hepatic excretion or by increased production of ALP by hepatic parenchymal or duct cells. Principle involved in estimation of alkaline phosphatase:

$$P\text{-nitrophenyl phosphate} + H_2O \xrightarrow[\text{Alkaline medium}]{AP} Phosphate\ ion + P\text{-nitrophenol}$$

ALP hydrolyses substrate P-nitrophenyl phosphate with the formation of P- nitrophenol and liberation of phosphate ion.

Serum Bilirubin
Estimation of bilirubin, metabolic product of the break down of heme is one of the better liver function tests. Normally, 0.25 mg/dl of conjugated bilirubin is present in the blood of an adult. Bilirubin level rises in diseases of hepatocytes, obstruction to biliary excretion into duodenum, in hemolysis and defects of hepatic uptake and conjugation of bilirubin treatment such as Gilbert's disease.[2] Bilirubin in serum reacts with diazo reagent in the presence of alcohol, after the proteins had been removed by precipitation.

Serum Protein
Liver cells synthesize albumin, fibrinogen, prothrombin, alpha-1-antitrypsin, haptoglobin, ceruloplasmin, transferrin, alpha foetoproteins and acute phase reactant proteins. The blood levels of these plasma proteins are decreased in extensive liver damage.

Morphological parameters
Morphological parameters like weight of the animals, weight and volume of the liver have also been used to evaluate the protective effect of the drug. Hepatotoxicity causes loss in liver weight/100 gm body weight of rats.

Hepatocyte Viability and Oxygen Uptake tests
Hepatotoxicants reduce the viability of hepatic cells as assessed by trypan blue exclusion and oxygen uptake tests. In liver, CCl_4 is metabolized to CCl_3O^- by cytochrome P-450 and the reactive oxidative free radical intermediate generated, O^- causes further damage. Utilization of oxygen by hepatocytes gets reduced; therefore the viability of hepatocytes is reduced.

Free Radical Scavenging
Free radicals are reactive molecules involved in many physiological processes and human diseases such as cancer, aging, arthritis, Parkinson syndrome, ischemia, toxin induced reaction, alcoholism, liver injury etc. The damage to hepatic parenchymal cells, leading to hepatic injury, is due to oxidative stress within the cells caused by partially reduced free oxygen (PRFO) species such as O_2 (Superoxide anion), H_2, O_2, and OH (hydroxyl free radical). The elevation of free radical levels seen during the liver damage is due to enhanced production of free radicals and decreased scavenging potential of the cells. A variety of intrinsic antioxidants (reduced glutathione, superoxide dismutase, glutathione- S-transferase etc.) are present in the organism, which protect them from oxidative stress.

Technically, the estimation of free radicals directly is not possible due to the transient nature of the free radicals. Thus estimations are usually done indirectly by measuring the "Antioxidant defense status" of the liver microsomes. Hepatoprotection by enzymatic free quenching is brought about by elevating the levels of antioxidant enzymes in tissues such as the Superoxide dismutase (SOD), Peroxidase and Catalase.
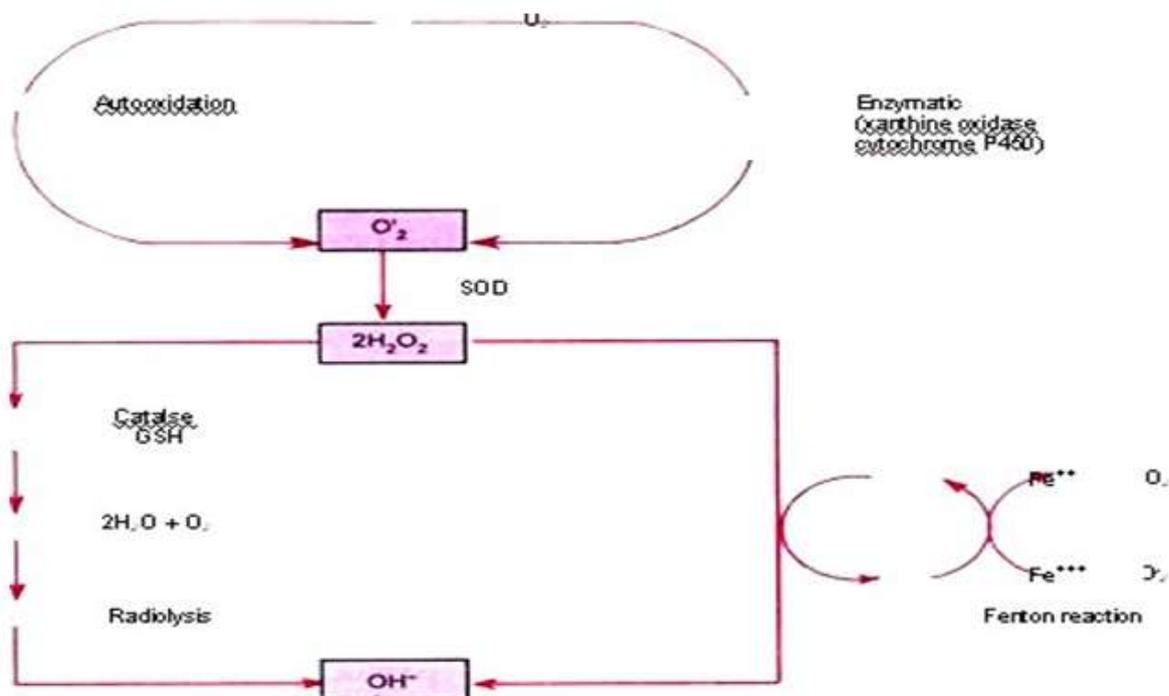
Figure-1: Mechanism of generation of free radicals.

The three partially reduced intermediate species between O_2 to H_2O are derived from enzymatic and nonenzymatic reaction as under:

1. Superoxide (O_2^-): superoxide anion O_2^- may be generated by direct autooxidation of O_2 during mitochondrial electron transport reaction. Alternatively O_2 is produced enzymatically by xanthine oxidase and cytochrome P_{450} in the mitochondria or cytosol. O_2 so formed is catabolised to produce H_2O_2 by superoxide dismutase.

2. Hydrogen peroxide (H_2O_2): H_2O_2 is reduced to water enzymatically by catalase (in the peroxisomes) and glutathione peroxidase GSH (both in the cytosol and mitochondria).

3. Hydroxyl radical: OH^- radical is formed by two ways in biologic processes-by radiolysis of water and by reaction of H_2O_2 with ferrous (Fe^{++}) ions, the latter process is termed as Fenton reaction.

Free radicals may produce membrane damage by the following mechanisms:

1. Lipid peroxidation: Polyunsaturated fatty acids (PUFA) of membrane are attacked repeatedly and severely by oxygen –derived free radicals to yield highly destructive PUFA radicals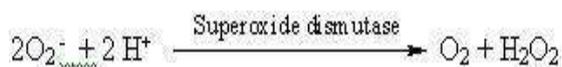 – lipid hydroperoxy radicals and lipid hydroperoxidation. The lipid peroxidase is decomposed by transition metals such as iron. Lipid peroxidation is propagated to other sites causing widespread membrane damage and destruction of organelles.

2. Oxidation of proteins: Oxygen- derived free radicals cause cell injury by oxidation of protein macromolecules of the cells, cross linking of labile amino acids as well as by fragmentation of polypeptides directly. The end result is degradation of cytosolic neutral proteases and cell destruction.

3. DNA damage: Free radicals cause breaks in the single strands of the nuclear and mitochondrial DNA. This results in cell injury; it may also cause malignant transformation of cells.

4. Cytoskeleton damage: Reactive oxygen species are also known to interact with cytoskeleton elements and interfere in mitochondrial aerobic phosphorylation and thus cause ATP depletion.

Superoxide dismutase (SOD):

SOD is a ubiquitous cellular enzyme, which dismutates superoxide radical to hydrogen peroxide and oxygen.[19] Dismutation is a reaction in which a single reactant is converted into two

different products. Superoxide dismutase, one of the chief cellular defense mechanisms, scavenges superoxide radicals by catalyzing the conversion of two of these radicals into hydrogen peroxide and molecular oxygen.

$$2O_2^- + 2H^+ \xrightarrow{\text{Superoxide dismutase}} O_2 + H_2O_2$$

Catalase

The hydrogen peroxide formed by superoxide dismutase and other processes is scavenged by catalase, a ubiquitous heme protein that catalyzes the dismutation of hydrogen peroxide into water and molecular oxygen. [20]

$$2H_2O_2 \longrightarrow H_2O + O_2$$

Glutathione (GSH)

GSH is a tripeptide of glycine, glutamic acid and cysteine. Glutathione is an important naturally occurring antioxidant as it prevents the hydrogen of sulfydryl group to be abstracted instead of methylene hydrogen of unsaturated lipids. Therefore, levels of glutathione are of critical importance in tissue injury caused by toxic substances. The antioxidant enzymes superoxide dismutase and glutathione form the first line of defense against free radical induced damage, offer protection against free radicals and thereby, maintain low levels of lipid peroxides. [10] The primary biological function of glutathione is to act as a non-enzymatic reducing agent to help keep cysteine thiol side chains in a reduced state on the surface of proteins. Glutathione is also used to prevent oxidative stress in most cells and helps to trap free radicals that can damage DNA and RNA.
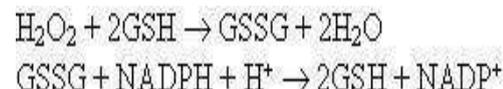
Lipid peroxidation

Lipid peroxidation can be defined as the oxidative deterioration of lipids. Lipid hydroperoxides are non-radical intermediates derived from unsaturated fatty acids, phospholipids, glycolipids, cholesterol esters and cholesterol itself. These are formed in enzymatic or non-enzymatic reactions involving free radical.

Glutathione peroxidase

Glutathione peroxidase (GPx) is an enzyme which catalyzes the reduction of hydroperoxides, including hydrogen peroxides, and functions to protect the cell from peroxidative damage. It is a selenium containing enzyme and reduces H_2O_2 to H_2O by oxidizing glutathione (GSH) Rereduction of the oxidized form of glutathione (GSSG) is then catalysed by glutathione reductase

$$H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O$$
$$GSSG + NADPH + H^+ \rightarrow 2GSH + NADP^+$$

GPx activity can be measured indirectly by a coupled reaction with glutathione reductase. Oxidized glutathione (GSSG) produced upon reduction of an organic hydroperoxide by GPx, is recycled to its reduced state by glutathione reductase and NADPH. The oxidation of NADPH to NADP+ is accompanied by a decrease in absorbance at 340 nm. The rate of decrease in the A340 is directly proportional to the GPx activity in the sample.

2.2.2 Assessment of Hepatoprotective activity Drugs and chemicals

Liv-52 (Himalayan Drug Co. Ltd. Mumbai), CCl4 (CDH, New Delhi), Olive oil (CDH, New Delhi), Carboxymethylcellulose (Himedia, Mumbai).

Experimental animals

Mature Male Albino Wistar rats weighing 180 – 280g were used for Pharmacological Studies. Animals were procured from Central Drug Research Institute, Lucknow and maintained at Central Animal Facility of the Institute. The animals were housed in Polypropylene cages under standard conditions of 27 ± 2^0 C temperature, 60-70% relative humidity and 12-12 hour light-dark period. The animals had free access to Standard rodent pellet diet and water ad libitum. The animals were acclimatized to lab conditions for 5 days prior to the experiment. Initial body weight of each animal was recorded. Each experimental group constituted six animals, housed in separate cages. Institutional Animal Ethical Committee (IAEC) had approved the experimental protocol (Regd. No. Bu/Pharm/IAEC/008/036). Care of animals was taken as per the guidelines of CPCSEA (Regd. No. 716/02/a/CPCSEA), Department of Animal Welfare, Government of India.

2.2.3 Acute toxicity studies

Acute Toxicity study of Carissa carandas Linn.[79] was performed in albino Swiss mice. The mice were kept for 4 hours of fasting prior to the experiment. The dose of 1ml/100g body weight was given orally to each mouse. The limit test for acute toxicity was performed at 5, 50, 100, 300 and

2000 mg/kg oral dose of Ethanolic extract of Carissa carandas (EECC) ;(OECD Guidelines 423, 2001).

During the first 4h after drug administration, animals were observed continuously for gross behavioral changes, and then observation is continued for 24h and 72h in regular intervals for 7 days. The parameters such as hyperactivity, grooming, convulsions, sedation, hypothermia, change in fur color, mortality or moribund state or death were observed.

2.2.4 Experimental design Doses and Treatments

Experimental protocol to evaluate the antihepatotoxic activity of Carissa carandas Linn. fruit against CCl4 induced hepatotoxicity was set according to Rao and Mishra (table 1) [80, 81] with slight modifications. The animals were divided into five groups with six animals in each group (n=6). Group I served as normal control which received 1ml normal saline (p.o.). Group II served as negative control which received normal saline (1 ml, p.o.) and CCl4 (1:1 CCl4 with Olive oil, 0.5 ml/kg body weight, i.v.) for 5 days. Group III served as standard group which received the Standard drug Liv-52 (0.125 mg/kg body wt., p.o) [59] for 15 days and CCl4 (1:1 CCl4 with Olive oil, 0.5 ml/kg body weight, i.v.) for 5 days. Group IV served as test 1 which received EECC 200 mg/kg body weight(p.o) for 15 days and CCl4 (1:1 CCl4 with Olive oil, 0.5 ml/kg body weight, i.v.) for 5 days. Group V served as test 2 which received only EECC 200 mg/kg body weight (p.o) for 15 days. EECC was suspended in sodium CMC in normal saline to make 10% suspension.

Sufficient quantity of food was replenished daily to the animals thrice in a day and access to water provided ad libitum during the experiment. Weight of each animal was recorded on every week.

2.2.5 Biochemical estimations

On the 16th day the animals were anesthetized under ether anesthesia and blood collected from retro orbital sinus using microcapillaries into eppendrop tubes. The animals were sacrificed by cervical dislocation. The animals were dissected out and the liver separated and blotted off blood through filter paper. The liver tissue was then washed in ice cold saline and preserved in 10% formasal solution for further estimations.

Serum was separated from the collected blood using centrifuge (Jyoti Labs, Gwalior) at 6000 rpm and subjected to biochemical estimations of different parameters like AST [82](Aspartate transaminase), ALT[85](Alanine transaminase), ALP[85](Alkaline phosphatase), SBLN[87](Serum bilirubin), TG[84](Triglycerides), TP[86] (Total protein) and ALBN[83](Albumin) using methods given in standard kits.

Liver was isolated from each dissected out animal. A portion of liver of each animal was washed with ice-cold saline, blotted off blood using blotting paper. Afterwards macerated with phosphate buffer (PH 7.4) and Liver homogenate was prepared by homogenizing the liver tissue in homogenizer (Jyoti labs, Gwalior) with 1000 rpm. Supernatant was used for the estimation of lipid profile (cholesterol, HDL, triglycerides) [84] and total protein[86].

Histopathological study

Liver separated and washed with normal saline, some portion of the liver was soaked in filter paper and transferred to 10% formalin solution in saline for histopathological study.

Procedure

1. **Dehydration**: liver pieces soaked for 15 minutes in absolute alcohol.
2. **Tissue embedding**: alcohol + xylene (1:1) for 20 minutes. Then mixture was concentrated off and pieces were put in xylene for 30 minutes. Scrapping of wax were added to the xylene up to saturation and kept for 24 hours.
3. **Paraffin infiltration and embedding**; the matured was filtered to remove any suspending particles and it was kept in molten state for 24 hours at 62^0-64^0C. The material was transferred directly in molten wax in the first infiltration pan for 45 minutes at 62^0C in oven. After the first embedding, tissue pieces were removed and placed in second infiltration and were kept as such at controlled temperature.
4. **Block preparation**: the lid of cuffing jar was applied on upper and side surface of lid. The filtered matured wax was poured in the lid up to 4/5th of the total height. The tissues were removed immediately from the infiltration pan and placed gently into the lid. The lid was placed in a tray containing water. It was kept as such till the block separated and floated in the water. The block was cut and trimmed to remove excess wax.
5. **Microtomy**: the block was then cut into ribbons of sections (5-7 μ) with the help of microtome. The ribbon sections were

transferred to a slide on which a fixative (egg albumin) had been applied previously.

6. **Staining of slides**: the sections on slides were dewaxed with xylol. Aqueous haematoxylin and alcoholic eosin dyes were used for staining. The sections were different concentrations of alcohol and xylol. Sections were mounted with Canada balsam on the slides carefully with glass rod, covered with coverslip, viewed and photographed.

2.2.6 Statistical analysis of data

The data were analyzed by GraphPad Prism 5 (GraphPad Software Inc; USA). The datas are presented as Mean ± SEM.

Student's t- test (normal control Vs toxic control) and One way ANOVA followed by Dunnett's post hoc test (Standard and Test Groups Vs Toxic control) were applied for determining statistical significance of difference in enzymes, protein and lipid levels between different groups.

The difference of $p < 0.05$ was considered significant in all the cases (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

III.   RESULTS AND DISCUSSION
Phytochemical screening

Various chemical test were performed for the assessment of carbohydrates, Proteins, amino acids, sterols, saponins, glycosides, flavonoids ,alkaloids and Phenolic content. Molish test revealed presence of carbohydrates, biuret and xanthoprotein test revealed presence of proteins, Ninhydrin test showed presence of amino acid, salkowaski and libermann buchard test showed presence of phenols, Keller-Killiani test and Borntrager's test suggested presence of flavonoids, Dragendroff and wagner's test suggest presence of alkaloids and lead acetate test suggest presence of Phenolic compound. Flavonoids, glycosides and phenolic compound suggest the hepatoprotctive effect of prunetin.

Table-1. Phytoconstituents present in Prunetin

S.No.	Chemical test	Observations	Inference
1.	Molish test	Violet colored ring appeared at the junction	Carbohydrates present
2.	Biuret test	Violet color appeared	Proteins absent
3.	Xanthoprotein test	White precipitate. turned orange on addition of NH4OH No purple color appeared	Proteins absent
4.	Ninhydrin test	Chloroform layer appeared	Amino acids absent
5.	Salkowski reaction	red and acid layer greenish yellow	Sterols Present
6.	Liebermann-Burchard test	First red, then blue and finally green color appeared	Sterols Present
7.	Saponification test	Soap was formed	Saponins Present
8.	Keller-Killiani test	Reddish brown color appeared at junction and bluish green at the upper layer	Glycosides Present
9.	Borntrager's test	Ammonical layer appeared pink	Glycosides Present
10.	Shinoda test	Pink color appeared	Flavonoids present
11.	Dragendroff's test	No buff precipitate was formed	Alkaloids Absent
12.	Wagner's test	No reddish-brown precipitate was formed	Alkaloids Absent
13.	Lead acetate test	Precipitate was formed	Phenolic compounds present

2. Experimental Protocol for testing liver protecting effect of Prunetin

There is 5 experimental groups to assess the hepatoprotective groups. Normal group will be treated with saline via oral route, control group will be treated with 0.5ml CCL4/kg body weight. Standard group will be treated with 0.5ml CCL4/kg body weight along with silymarin at a dose 50mg/kg daily. Treatment group 1 will be treated with Prunetin at a dose 20 mg/kg and 0.5 ml CCL4/kg body weight. Treatment group 2 will be treated with prunetin at a dose of 40mg/kg along with 0.5 ml CCL4/kg body weight. After 5 days of treatment, some animals will be sacrificed and further, various biochemical parameters will be assessed and histopathological studies will be performed.

Table-2: Experimental Protocol for Testing Liver Protecting Effect of Prunetin

Treatment	Dose, Route and length of Treatment1
Saline (Normal)	1 ml saline orally daily for 5 consecutive days.
CCl4 + saline (Control)	0.5 ml CCl4/kg body wt. i.v. + 1ml saline orally daily for 5 consecutive days.
Silymarin + CCl4 (Standard)	0.5 ml CCl4/kg body wt. i.v. daily for 5 consecutive days daily + Silymarin 50 mg/kg daily, for 15 consecutive days, beginning 3 days after last CCl4 treatment.
Prunetin (20 mg/kg) + CCl4 (Treatment group 1)	20 mg/kg body wt. orally for 1 day and then extract + CCl4 0.5 ml/kg body wt. for 5 consecutive days followed by extract for 15 days after last treatment of CCl4
Prunetin (40 mg/kg) + CCL4 (Treatment group 2)	40mg/kg body wt. orally daily for 15 consecutive days

3. Pharmacological evaluations Acute toxicity studies

Acute toxicity was performed at 5, 50, 100, 300 and 2000 mg/kg oral dose of Prunetin in group of three mice (OECD 423 guidelines, 2001). The mice exhibited normal behavioral, neurological and autonomic profiles. No mortality was observed at limit dose of 2000mg/kg body weight (p.o.) for the duration of 72 hours and thereafter up to seven days observation. Thus, the safe dose for experimental work is 1/100th of limit dose i.e. is 20 mg/kg body weight.

4. Assessment of hepatoprotective activity

1. **Effect of Prunetin on Biochemical Parameters (SGOT, SGPT ALP, SBLN) in Serum in CCL4 induced hepatotoxicity:** Control group showed increase in SGOT ($p < 0.05$), SGPT ($p < 0.05$), ALP ($p < 0.05$) and SBLN ($p < 0.05$) as compared to normal group. Silymarin treated group showed decrease in the level of SGOT ($p < 0.05$), SGPT ($p < 0.05$), ALP ($p < 0.05$) and SBLN ($p < 0.05$) as compared to control group. Moreover, 15 days treatment of Prunetin at two different doses reversed the condition. Prunetin 20mg/kg and Prunetin 40 mg/kg decreased the level of SGOT ($p < 0.05$), SGPT ($p < 0.05$), ALP ($p < 0.05$) and SBLN ($p < 0.05$) as compared to control group. Moreover, Prunetin at the dose 40 mg/kg exhibited more pronounced effect.

Table-3: Effect of Prunetin on Biochemical Parameters (SGOT, SGPT ALP, SBLN) in Serum

Group	Biochemical parameters(a)			
	SGOT (IU/L)	SGPT (IU/L)	ALP (IU/L)	SBLN (mg/dl)
Group I Saline (Normal)	19.98 ± 0.30	16.505± 1.465	43.295± 1.734	0.77± 1.27
Group II CCl4 + saline	25.81± 0.93(b)***	26.187± 3.095(b)*	74.486 ± 7.44(b)**	1.03± 3.56(b)**

(Control)				
Group III Silymarin+ CCl4	20.835± 0.749(c) ***		17.876± 1.58(c)*	52.878± 4.718(c)*	1.11± 6.08(c)*
Group IV Prunetin (20 mg/kg)+ CCl4	19.986± 0.77(c)***		16.01± 2.44(c)**	49.808± 3.846(c)**	0.97± 0.50(c)**
Group V Prunetin (40 mg/kg) only	19.835± 0.756(c)***		17.925± 1.054(c)*	45.23± 2.707(c)***	1.09± 0.87(c)*

a.   Values are mean+-SEM of 6 animals in each group.
b.   Values are $p < 0.05$ as compared to group I.
c.   Values are $p < 0.05$ as compared to group II.

2. Effect of Prunetin on biochemical parameters (TP, ALB, TG) in serum in CCL4 induced hepatotoxicity

Control group showed that there is decrease in TP ($p < 0.05$), ALB ($p < 0.05$), TG ($p < 0.05$) as compared to normal group. Silymarin treatment group revealed increase in TP ($p < 0.05$), ALB ($p < 0.05$), TG ($p < 0.05$) as compared to control group. Moreover 15 days treatment of Prunetin at two different doses upturned the situation. Prunetin 20mg/kg and Prunetin 40 mg/kg decreased the level of TP ($p < 0.05$), ALB ($p < 0.05$), TG ($p < 0.05$) as compared to control group. Moreover, Prunetin at the dose 40 mg/kg showed more marked effect (Table 4).

Table-4: Effect of Prunetin on Biochemical Parameters (TP, ALB, TG) in Serum

Groups	Biochemical parameters(a)		
	TP (g/100g)	ALB (g/dl)	TG (mg/dl)
Group I Saline (Normal)	5.63±0.15	6.73±0.21	19.88±0.35
Group II CCl4 + saline (Control)	3.9±0.22(b)*	3.105±1.485(b)*	38.10±0.59(b)***
Group III Silymarin + CCl4	4.76±0.24(c)*	6.082±0.36(c)***	25.925±2.08(c)*
Group IV Prunetin (20mg/kg)+ CCl4	6.1±1.67(c)*	6.26±0.35(c)***	19.73±0.26(c)***
Group V Prunetin (40mg/kg) only	5.4±0.18(c) *	6.31±0.32(c)***	19.57±0.74(c)***

a.   Values are mean+-SEM of 6 animals in each group.
b.   Values are $p < 0.05$ as compared to group I.
c.   Values are $p < 0.05$ as compared to group II.

3. Effect on Prunetin on HDL, Cholesterol and TP in liver in CCL4 induced hepatotoxicity

In liver homogenate also, the level of HDL, Cholesterol, Triglyceride were found significantly lower in toxic controls as compared to

normal group (table 5, p<0.05). Rise in HDL, cholesterol, triglycerides and total protein was observed in these groups much higher (p<0.05) as compared to toxic controls. Prunetin at two different doses (20 mg/kg) and (40 mg/kg) upturned the condition. Prunetin at (20 mg/kg) decreased the level of HDL (P<0.05), Cholesterol (P<0.05), Triglycerides (P<0.05) and TP (P<0.05) as compared to control group, Pruetin at 40 mg/kg decreased the level of HDL, Cholesterol, Triglycerides and TP (P<0.05) as compared to control group. At these two groups, Prunetin at 40 mg/kg showed most pronounced effect than prunetin at 20 mg/kg dose. Prunetin at 40 mg/kg showed the hepatoprotective effect more than the silymarin treatment group.

Table-5 Effect of Prunetin on Biochemical Parameters (HDL, Cholesterol, Triglycerides, TP) in Liver in CCL4 induced hepatotoxicity

Group	Biochemical parameters(a)			
	HDL	Cholesterol	Triglyceride	TP
Group I Saline (Normal)	55.62± 3.22	223.7± 1.69	35.63± 0.38	8.11± 0.12
Group II CCl4 + saline (Control)	60.23± 6.08 (B)**	318.63± 0.38(B)**	29.57± 4.11(B)***	5.72± 0.13(B)**
Group III Silymarin + CCl4	57.22± 1.33(C)**	180.17± 3.56(C)*	24.75± 0.87(C)***	6.86± 0.26(C)*
Group IV Prunetin (20 mg/kg) + CCl4	47.23± 0.23(C)***	187.64± 0.20(C)**	21.22± 2.14(C)***	7.77± 0.21(C)**
Group V Prunetin (40 mg/kg)+ CCl4	52.69± 0.33(C)**	169.45± 1.33(C)***	29.13± 3.09(C)***	6.11± 0.46(C)**

A. Values are mean ± SEM of 6 animals in each group.
B. Values are p<0.05 as compared to group I.
C. Values are p<0.05 as compared to group II.

Histopathological Study
After paraffin embedding and block making, serial sections of 5µ thickness were made, stained with Ehrlich's Haematoxylin and Eosin and examined under Research Binocular with digital camera (MP 5100). A few photomicrographs of representative types are given here histopathological study of liver tissue revealed the protective effect of prunetin. Histopathological studies showed about the changes in liver structure. It determines whether the treatment improves the changes in fatty changes or not. Photomicrograph
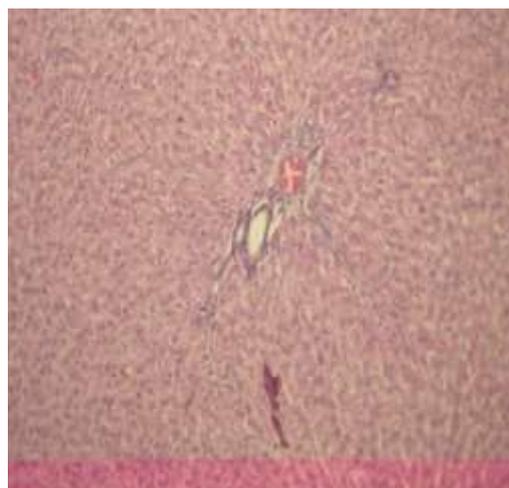

Figure 5 (1*10x). Photomicrograph of Group I animals. Showing normal histopathology of liver cells.
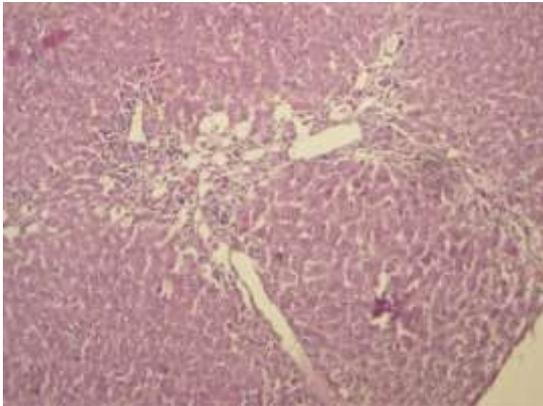
Figure 6 (1*10x). Photomicrograph of Group II animals.

Showing fatty changes, central vein necrosis along with centrizonal fatty change.

Figure 7 (1*10x). Photomicrograph of Group III animals.
Necrosis still persists with less in severity, showing healing effect.
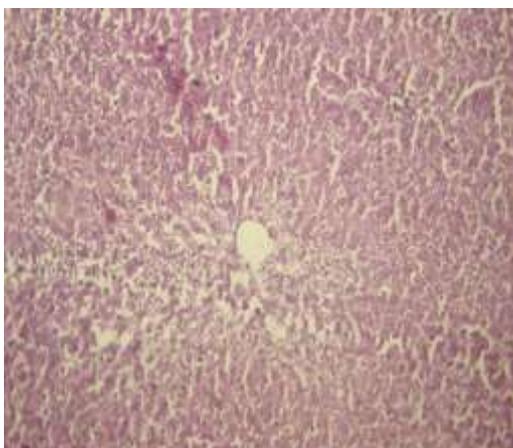

Figure 8 (1*10x). Photomicrograph of Group IV animals.

Fatty change can be seen; preventory infiltration and healing of central vein necrosis can be seen. Histopathology is almost similar to normal group (group I).
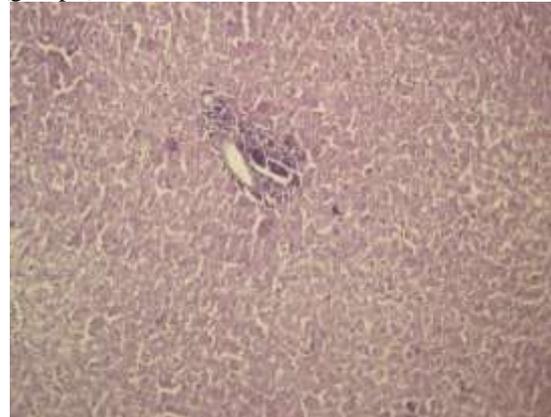

Figure 9 (1*10x). Photomicrograph of Group V animals.
Fibrosis, preventory infiltration are observed. Fatty change not seen.
Histopathology is almost similar to normal group (group I).

Histopathological images of different group showed different results. In normal group Showing normal histopathology of liver cells. Control group showed fatty changes, central vein necrosis along with centrizonal fatty change. Photomicrograph of Group III animals showed necrosis still persists with less in severity, showing healing effect. Photomicrograph of Group IV animals showed fatty change preventory infiltration and healing of central vein necrosis can be seen and the result shows similar to normal group. Fatty change can be seen; preventory infiltration and healing of central vein necrosis can be seen. Histopathology is almost similar to normal group (group I). Photomicrograph of Group IV animals showed fatty changes, preventory infiltration and healing of central vein necrosis can be seen. Histopathology is almost similar to normal group (group I). Photomicrograph of Group V animals showed Fibrosis, and preventory infiltration. Fatty changes are not seen. Histopathology is almost similar to normal group (group I). Among the two-treatment group prunetin at 40 mg/kg showed better results than prunetin at 20 mg/kg dose. Both the treatment group showed results similar to standard group that is silymarin. Histopathology studies indicated that prunetin at 40 mg/kg is the correct dose to reverse the ill effects on liver.

IV. CONCLUSION

Hepatoprotective activity of several herbal extracts has been previously tested using CCl4 rat liver injury model(Ugwu and Suru 2021). The rational of the present study is to scientifically authenticate and claim the hepatoprotective activity of Prunetin. Prunetin is an O-methylated flavonoid that is present in various natural plants and a primary significant compound found in isoflavone (Patel and Patel 2022). Prunetin has many therapeutic actions but its action in hepatoprotetive is unexplored. As there is very few hepatoprotective agents available till date, therefore our study mainly focused on to explore the therapeutic potential of prunetin as a hepatoprotective agent.

Two doses of prunetin were selected for the study 20 mg/kg and 40 mg/kg. Silymarin was taken as standard drug to compare the effects of prunetin as hepatoprotective agents. The results have proved significant hepatoprotective activity of Prunetin (20 mg/kg) and Prunetin (40mg/kg). The relatively high LD50 value obtained in this study of prunetin suggests that the extract is relatively safe and non-toxic to rats. Acute toxicity study suggested that two doses of prunetin were found safe. Therefore, these two doses were selected for the present investigation. Plethora of literature suggested that activity of liver protection is possibly due to presence of flavonoids, terpenes, glycosides, anthocyanins, alkaloids, antioxidants etc., these constituents have well established potential as antioxidant, anti-inflammatory and anti-fibrotic (Sahu, Goswami et al. 2023). All these properties related to prunetin make the prunetin a very good therapeutic potential for liver protective agent.

Carbontetrachloride is a standard hepatotoxin used in animal models. The changes induced by CCl4 in liver are similar to that of acute viral-hepatitis. Metabolic activation and hepatotoxic mechanism of CCl4 is (i) Cytochrome P-450-dependent reduction of CCl4 to CCl3 free radical (ii) formation of CCl300 free radical (peroxyl radical) by reaction with oxygen molecule. (iii) Both CCl3 and CCl300 free radical can induce an H abstraction from unsaturated fatty acids (iv) in this process, they become saturated by the formation of CHCl3 or CCl300H(v) conversion of carbon centered lipid radical to conjugated aldehyde (MDH). Summarily these changes elicit lipid peroxidation, disturb calcium homeostasis, which alter functional integrity of liver and finally result in cell death (Boll, Weber et al. 2001).

Result of present study suggested that in prunetin carbohydrates, Proteins, amino acids, sterols, saponins, glycosides, flavonoids, alkaloids and Phenolic content were present.

Among all flavonoids, glycosides and phenolic compound are responsible for the hepatoprotctive effect of prunetin.

Further acute toxicity study was performed to assess the correct dose of prunetin which is not toxic. Acute toxic study suggested that 20mg/kg and 40 mg/kg dose is the safe dose.

Animals were divided into five groups and various protocols were performed to assess the hepatoprotective effect. Normal group was treated with normal saline. Control group was treated with 0.5ml CCL4/kg body weight. Standard group was be treated with 0.5ml CCL4/kg body weight along with silymarin at a dose 50mg/kg daily. Treatment group 1 was treated with Prunetin at a dose 20 mg/kg and 0.5 ml CCL4/kg body weight. Treatment group 2 was treated with prunetin at a dose of 40mg/kg along with 0.5 ml CCL4/kg body weight.

Estimating the activities of serum marker enzyme like SGOT, SGPT ALP, SBLN (Yadav, Chanda et al. 2010, Datta, Aggarwal et al. 2023) and total protein can make assessment of liver function (Himel 2020, Sharma 2022).When liver cell plasma membrane is damaged, a variety of enzymes normally located in the cytosol are released into the blood stream (Abou Seif 2016). Their estimation in serum is a useful quantitative marker of the extent and type of hepatocellular damage, for e.g. elevated AST and ALT is indicative of hepatocyte necrosis, elevated ALP indicates cholestasis and elevated bilirubin in serum indicates metabolic defects of liver. Hepatic damage as evidenced by a rise in the levels of AST, ALT, ALP, TP and total lipid in serum and also changes observed in other biochemical parameters in serum and liver showed a tendency to attain near normal values in animals co-administered with silymarin. The enhanced activity of these enzymes observed in Group II corresponds to the extensive liver damage induced by the toxin carbontetrachloride. The normal enzyme levels in Group III to V rats is a clear indication of hepatoprotective effect of Prunetin. In control group TP, ALP and TG value were decreased than normal group which suggest hepatotoxic action in the control group. Further, increase in the value of TP, ALB and TG in group 4 and group 5 suggest hepatoprotective action and there is a prominent increase in the level of TP, ALP and TG level that suggest good hepatoprotective action. Among these

two-treatment group 5 shows best hepatoprotective action.

The values for HDL, Cholesterol, triglycerides and AST in control group were decreased in normal group due to hepatotoxic actions of CCL4. Further reversal of the condition is due to treatment with silymarin and prunetin at two different doses. Group 4 and group 5 showed prominent effect as hepatoprotective effect. Among all the treatment groups group 5 showed best effect.

Profound steatosis, ballooning degeneration and nodule formation was observed in the hepatic architecture of CCl4 treated rats which were found to acquire normal architecture in EECC treated animals. A comparative histopathological study of liver cell from different groups further corroborated the hepatoprotective effect of Prunetin. However more elaborate work is required to establish the effect of Prunetin as potential hepatoprotective.

In conclusion, Prunetin exhibited hepatoprotective activity and may be of potential to treat liver damages like, fatty liver, cholestasis, hepatic necrosis and metabolic defects etc. the overall protection can be assumed due to the enhanced immunity of the body by Prunetin.

The obtained result justifies the title of the project. Fruit extract exhibited prominent hepatoprotective effect. The hepatoprotective activity of EECC can be attributed to phytochemicals like triterpenoids, flavonoids, saponins, anthocyanins, alkaloids and sterols.

The present study suggests the use of Carissa carandas Linn. fruit either alone or with other active hepatoprotective herbs to alleviate liver disorders. However more elaborative work is required to establish efficacy of the drug and to ascertain the complete chemical constituents of the plant and isolation and elucidation of mechanism of action of these ingredients in liver protection. The safety profile of the fruit extracts as a medicinal remedy for liver disorders are to be studied extensively.

However, these are preliminary pilot studies but more extensive preclinical and clinical trail must be conducted to establish the role of prunetin as a hepatoprotective effect.

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