EVALUATION OF *IN VITRO* AND *IN VIVO* HEPATOPROTECTIVE ACTIVITY OF *Pongamia pinnata* Linn., SEED EXTRACT AGAINST CARBON TETRACHLORIDE INDUCED HEPATOTOXICITY IN WISTAR RATS

A dissertation submitted to

THE TAMILNADU DR.M.G.R MEDICAL UNIVERSITY

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in partial fulfilment of the requirements for the award of the degree of

MASTER OF PHARMACY
IN
PHARMACOLOGY

Submitted by
Reg. No. **261426061**

Under the guidance of
Dr. N. JAYASHREE, M.Pharm., Ph.D.,

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CHENNAI - 600003

APRIL 2016

CERTIFICATE

This is to certify that the dissertation entitled "**EVALUATION OF *IN VITRO* AND *IN VIVO* HEPATOPROTECTIVE ACTIVITY OF *Pongamia pinnata* Linn., SEED EXTRACT AGAINST CARBON TETRACHLORIDE INDUCED HEPATOTOXICITY IN WISTAR RATS"** submitted by the **Reg. No. 261426061** in partial fulfilment of the requirements for the award of the degree of **Master of Pharmacy in Pharmacology** by the Tamil Nadu Dr.M.G.R Medical University, Chennai, is a bonafide work done by her during the academic year 2015-2016 under the guidance of **Dr. N. Jayashree, M.Pharm., Ph.D.,** Professor of Pharmacology, Institute of Pharmacology, Madras Medical College, Chennai-03.

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CONTENTS

LIST OF ABBREVATIONS

PPE - *Pongamia pinnata* extract

SOD - Superoxide dismutase

CAT - Catalase

LPO - Lipid peroxidation

GPx - Glutathione Peroxidase

AST - Aspartate Aminotransferase

ALT - Alanine Aminotransferase

ALP - Alkaline phosphatase

TB - Total Bilirubin

TP - Total protein

ALB - Albumin

IFN-γ - Interferon- γ

VCAM-1 - Vascular Cell Adhesion Molecule1

ICAM-1 - Inter Cellular Adhesion Molecule1

PECAM - Platelet Endothelial Cell Adhesion Molecule1

IL - Interleukin

TNF ∝ - Tumor Necrosis Factor ∝

CBC - Complete Blood count

ERCP - Endoscopic Retrograde Cholangio Pancreatography

MRI - Magnetic Resonance Imaging

CT - Computerized Tomography

HRBC - Human Red Blood Cells

TBA - Thiobarbituric acid

EE - Ethanol Extract

HPLC - High Performance liquid Chromatography

MS - Mass spectroscopy

GC-MS - Gas chromatography-mass spectroscopy

NMR - Nuclear Magnetic Resonance Spectroscopy

1. INTRODUCTION

The liver is a major detoxifying organ in the body. It is also one of the largest organs in the human body and is a intense site for metabolism. It has a major role in the maintenance, performance and regulation of homeostasis of the body. It is involved with almost all the biochemical pathways responsible for growth, nutrient supply, energy provision and reproduction. The major functions of the liver are carbohydrate, protein and fat metabolism, detoxification, secretion of bile and storage of vitamins.

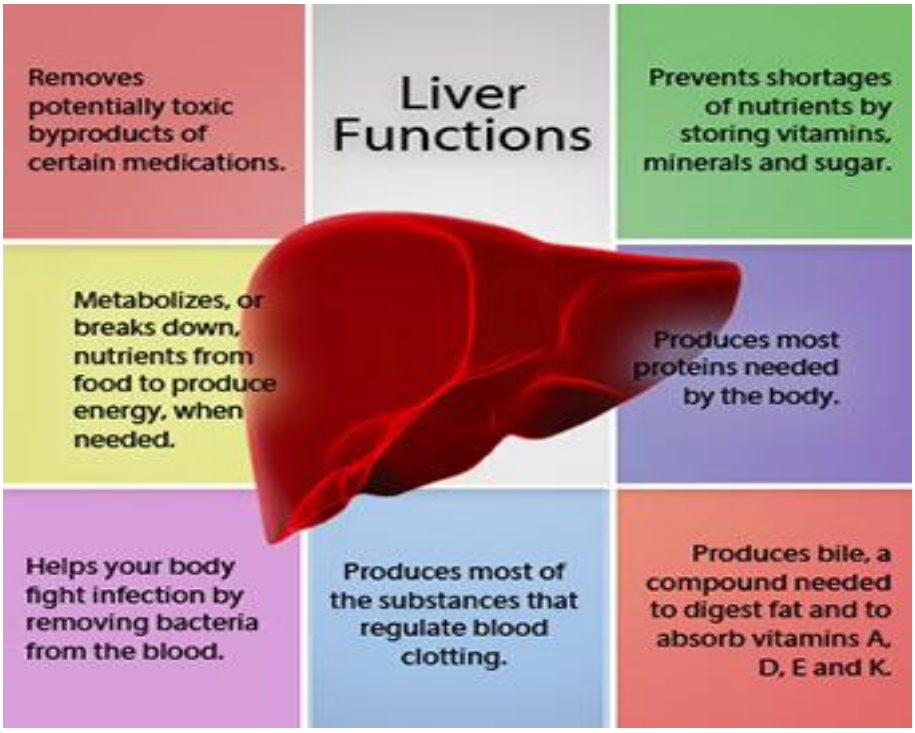


Figure . 1 Functions of liver

Liver diseases are posing as a major health problem around the world. Toxic industrial chemicals, alcohol, viral infection, water pollutants and aflatoxins are major risk factors of liver disease[1].

There are more than a hundred kinds of liver diseases, some of the most common ones are[2],

1. **Hepatitis,** inflammation of the liver, is caused by various viruses (viral hepatitis) liver toxins (e.g. alcoholic hepatitis), autoimmunity (autoimmune hepatitis) or hereditary conditions.
2. **Alcoholic liver disease** is a hepatic manifestation of alcohol over consumption. It includes fatty liver disease, alcoholic hepatitis and cirrhosis.
3. **Fatty liver disease** is a reversible condition where large vacuoles of triglyceride fat accumulate in liver cells.
4. **Non-alcoholic fatty liver disease** is a spectrum of disease associated with obesity and metabolic syndrome.
5. **Hereditary diseases** that cause damage to the liver include hemochromatosis, involving accumulation of **iron** in the body.
6. **Gilbert's syndrome**, a genetic disorder of **bilirubin** metabolism found in a small percent of the population, can cause mild **jaundice.**
7. **Cirrhosis** is the formation of fibrous tissue (fibrosis) in the place of liver cells that have died due to a variety of causes, including viral hepatitis, alcohol over consumption and other forms of liver toxicity. Cirrhosis causes chronic liver failure.
8. **Autoimmune disorders** Sometimes the immune system may begin to attack the liver or bile ducts causing inflammation and scarring which leads to a progressive form of liver disease. Examples of liver diseases believed to be caused by the immune system are primary biliary cirrhosis (PBS), primary sclerosing cholangitis (PSC) and autoimmune hepatitis.
9. **Fascioliasis,** a parasitic infection of liver caused by a Liver fluke of the Fasciola genus, mostly the *Fasciola hepatica*.

Along with the major health complications such as cancer, respiratory diseases, cardiovascular diseases, the incidence of **liver disease** is also on the rise along with the growing population. It is a major cause of death increasing every year and it is the fifth big killer in countries such as England and Wales.

According to the latest WHO data published in May 2014, liver disease deaths in India reached 216,865 or 2.44% of total deaths[3]. The worldwide death from cirrhosis and liver cancer is 50 million per year. 1.3 million deaths worldwide are due to chronic viral hepatitis. The harmful use of alcohol results in 2.5 million deaths worldwide each year with over 5,000 deaths in England and Wales in each of the last ten years[4]. Statistics obtained from birth and death registration department of Pune Municipal Corporation, India showed that an average of 35-40 people die every month from liver related problems. Contrary to popular belief, even non-alcoholics can fall to deadly liver diseases. Alcohol abuse continues to cause maximum cases of liver cirrhosis [5].

CAUSES OF LIVER DAMAGE[6, 7]

Chemical induced liver damage: Carbon tetrachloride, alcohol consumption, aflatoxins, 1, 1, 2, 2- tetrachloroethane, carbon tetrabromide, dimethyl formamide, ethylene dichloride.

Alcohol abuse

Drug induced: More than 900 drugs have been implicated in causing liver injury and it is the most common reason for a drug to be withdrawn from the market. Drug induced liver injury is responsible for 5% of hospitalised persons and 50% of all acute liver failures. Some of them are Acetaminophen over dose, Amiodarone, Ketoconazole, Rifampicin,

Virus induced: Hepatitis A, B, C, D and E.

Other causes: Non-alcoholic fatty liver, malnutrition, extrahepatic infections, ingestion of poisonous wild mushrooms, haemochromatosis.

PATHOPHYSIOLOGY OF LIVER DAMAGE

The hepatocellular stress induced by hepatotoxins or by viruses may lead to the activation of liver resident macrophages on one side and to the release of chemokines on other side. Proinflammatory cytokines are Interleukins-1β, Interferon-gamma (IFN-γ), whose tissue concentration increases early after toxins administration, followed by Tumor Necrosis Factor-α, Interleukin-6 in a similar kinetics, which are released by natural killer cells as well as kupffer cells.

Figure. 2 Pathophysiology of liver damage

They induce an increase expression of cell adhesion molecules like ICAM-1 and VCAM-1 on the portal or sinusoidal endothelial cells and a down regulation of PECAM 1.These molecules allow the recruitment and sinusoidal transmigration of inflammatory cells toward their target, the hepatocyte.

Inflammation perpetuates as long as damaging noxae remain present or are repeatedly administered. Leukocytes may enter the liver tissue mainly enter the portal tract, where the inflammation mainly initiates[8].

MECHANISM OF LIVER DAMAGE[7, 9]

75% of blood coming to the liver arrives directly from gastrointestinal organs and then spleen via portal veins that bring drugs and xenobiotics in near-undiluted form. Several mechanisms are responsible for either inducing hepatic injury or worsening the damage process. Many chemicals damage mitochondria, an intracellular organelle that produces energy. Its dysfunction releases excessive amount of oxidants that, in turn, injure hepatic cells. Activation of some enzymes in the cytochrome P-450 system such as CYP2E1 also leads to oxidative stress. Injury to hepatocyte and bile duct cells lead to accumulation of bile acid inside the liver. This promotes further liver damage. Non-parenchymal cells such as Kupffer cells, fat storing stellate cells, and leukocytes (i.e. neutrophil and monocyte) also have a role in the mechanism.

Immune mechanisms involve cell cooperation and are mediated by cytokines, nitric oxide and complement. Pathologic apoptosis is potentially an important mechanism of acute liver injury. Specific attention is paid here to the more frequent causes of acute liver failure, hypoxia/reoxygenation, liver congestion, acetaminophen poisoning, posttransplant acute liver rejection, severe sepsis, viral hepatitis, and alcoholic liver disease. Knowledge of the intimate mechanisms of liver injury at the cellular level may lead to adaptation of therapeutic strategies that will prevent end-stage liver failure.

STAGES OF LIVER DAMAGE

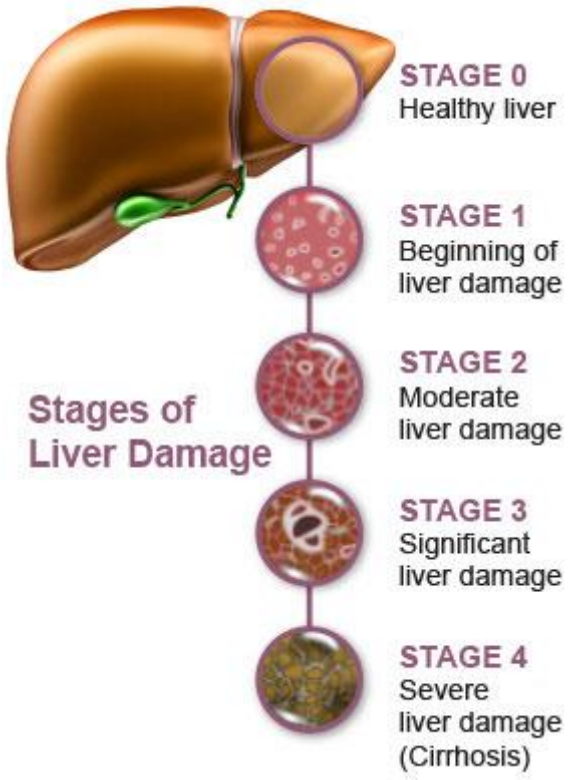


Figure. 3 Stages of liver damage

SIGNS AND SYMPTOMS OF THE LIVER DISEASE[9, 10]

Symptoms partly depend on the type and the extent of liver disease. In many cases, there may be no symptoms. Signs and symptoms that are common to a number of different types of liver disease include,

- Jaundice or yellowing of the skin
- Darkened urine
- Nausea, vomiting
- Loss of appetite
- Unusual weight loss or weight gain

- Diarrhoea
- Light-coloured stools
- Abdominal pain in the upper right part of the stomach
- Generalized itching
- Varicose veins (enlarged blood vessels)
- Hypoglycaemia (low blood sugar)
- Muscle aches and pains
- Depression

RISK FACTORS

A number of factors increase the risk of developing liver disease, which can lead to liver failure. Risk factors include,

- Alcoholism
- Coronary artery disease (due to atherosclerosis or hardening of the arteries, or other causes)
- Diabetes
- Exposure to certain toxins, such as arsenic
- Exposure to hepatitis
- High triglyceride levels in the blood
- Long-term treatment with corticosteroids
- Obesity

DIAGNOSIS

A healthcare professional can determine whether a person symptoms, medical history, and physical exam are consistent with liver disease. Hepatomegaly, an enlarged, firm liver, and other signs of liver disease may be found on examination.

Many further tests may also be used to support the diagnosis. These include blood tests, such as

- Liver function tests, which are blood tests that check a wide variety of liver enzymes and by products.
- A complete blood count (CBC), which looks at the type and number of blood cells in the body.
- Abdominal X-rays.
- Ultrasounds, to show size of abdominal organs and the presence of masses.
- An upper GI study, which can detect abnormalities in the oesophagus caused by liver disease.
- Liver scans with radio tagged substances to show changes in the liver structure.
- ERCP or Endoscopic Retrograde Cholangio Pancreatography. A thin tube called an endoscope is used to view various structures in and around the liver.
- Abdominal CT scan or abdominal MRI, which provide more information about the liver structure and function.

In some cases, the only way to definitively diagnose the presence of certain liver diseases is by a liver biopsy. This procedure involves the removal of a tiny piece of liver tissue for examination under a microscope. Liver biopsies may have to be done repeatedly to see how the disease is progressing or responding to treatment.

Some of the drugs currently available in the management of liver disease are,

1) Ursodeoxycholic acid (Ursodiol)

Mechanism of action

It is more hydrophilic and hepatotoxic than the major circulating bile acids in humans. The immunomodulatory effects of Ursodeoxycholic acid are to involve decreased immunoglobulin production by B lymphocytes, decreased interleukin-1 and interleukin-2 production by T lymphocytes, decreased expression of hepatocyte cell surface membrane HLA Class I molecules and possibly stimulation of the hepatocyte glucocorticoid receptor.

Clinical applications

Ursodeoxycholic acid has been used in the management of chronic hepatic diseases in humans such as primary biliary cirrhosis, biliary disease secondary to cystic fibrosis, non-alcoholic steatohepatitis, idiopathic chronic hepatitis, autoimmune hepatitis, primary sclerosing cholangitis, and alcoholic hepatitis.

Dose rate

It is recommended that Ursodeoxycholic acid be administered for 3-4 months after which the patient should be reassessed for improvement in biochemical markers of hepatocellular pathology. If there has been improvement, treatment is continued, but if there has been no improvement or progression, either treatment should be terminated or additional therapies such as glucocorticoids or colchicine added.

Adverse effects

Diarrhoea, Vomiting

2) Penicillamine

Penicillamine is a degradation product of penicillin but has no antimicrobial activity. It was first isolated in 1953 from the urine of a patient with liver disease who was receiving penicillin.

Penicillamine is a monothiol chelating agent which is used in veterinary medicine in the treatment of copper-storage hepatopathy (e.g. Bedlington Terriers), lead toxicity and cystine urolithiasis. It has also been used in the management of rheumatoid arthritis and Wilson's disease.

Dosage and formulations

For management of copper-associated hepatopathy, a dose of 10–15 mg/kg q12h p.o is given.

Adverse effects

- GIT adverse effects are common resulting in nausea and vomiting. Smaller doses on a more frequent basis may alleviate adverse effects. Alternatively, the drug can be given with food although this will reduce absorption.

Other adverse effects observed infrequently or rarely include,

- Fever
- Lymphadenopathy
- Skin hypersensitivity reactions
- Immune-complex glomerulonephropathy

Other drugs include

Liver disease treatment will depend on the type and the extent of disease. For example, treating hepatitis B, hepatitis C and hepatitis D may involve the use of medications such as the antiviral medication alpha interferon. Other medications used to treat liver disease may include ribavirin, lamivudine, steroids and antibiotics.

The currently available drugs, though useful, are also associated with side effects.

IMPORTANCE OF HERBS IN TREATING LIVER DISORDER

Herbal medicines are in great demand in the developed world due to their efficacy, safety and lesser side effects. Herbal drugs are most widely used than allopathic drugs as hepatoprotective because they are inexpensive, have better cultural acceptability, better compatability with the human body and minimal side effects.

A number of plants and traditional formulations are available for the treatment of liver diseases. Around 170 phytoconstituents isolated from 110 plants belonging to 55 families have been reported to possess hepatoprotective activity[11]. It is estimated that about six hundred (600) commercial herbal formulations are used world over as hepatoprotective drugs.

Amongst the many plants that have hepatoprotective activity, Silymarin is widely used as a hepatoprotectant around the world. It is a mixture of flavanoid complexes and is the active component of milk thistle plant that protects liver and kidney cells from toxic effects of drugs, including chemotherapy[12]. Silymarin is composed of a mixture of four isomeric flavonolignans: silibinin, isosilibinin, silydianin and silychristin. Silymarin possesses free radical scavenging ability, it increases antioxidant enzymes, such as superoxide dismutase (SOD) and catalase and inhibits lipid peroxidation. It is reported to offer protection against various chemical hepatotoxins such as CCl_4 and alcoholic liver.

Many plants like *Solanum xanthocarpum, Adiantun incisum, Ardisis solanacea Parmelia perlata*, etc. have been reported to possess hepatoprotective activity against CCl_4 induced hepatotoxicity. Some lichens like *Usnea ghattensis* have been reported to contain antioxidant and hepatoprotective activity against ethanol induced liver damage[13].

There are many plants which have not been subjected to a through scientific evaluation. One such plant is *Pongamia pinnata* L., In this plant the leaves and stem bark have been studied and they are known to possess hepatoprotective activity. These are rich in flavonoids such as furanoflavones, furanoflavonols, chromenoflavones, furanochalcones and pyranochalcones and it consist of several flavone and chalcone derivatives such as Pongone, Galbone, Pongagallone A and B[14]. The presence of flavonoids has been reported as the reason for their hepatoprotective activity.

The seeds have not been so far subjected to hepatoprotective activity screening. The seeds of *Pongamia pinnata* L., are also rich in flavonoids. Hence the present study was carried out to explore the hepatoprotecive efficacy of seeds of *Pongamia pinnata* L., against CCl_4 induced hepatotoxicity in rats model.

2. REVIEW OF LITERATURE

2.1. PHARMACOLOGICAL STUDIES

- **Kaur *et al.*, 2014** evaluated the hepatoprotective activity of the ethanolic and aqueous stem bark extract of *Pongamia pinnata* L., against paracetamol induced hepatotoxicity. The efficacy of protection was measured by evaluation of biochemical parameters, such as SGOT (Serum glutamate oxalate transaminase), SGPT (Serum glutamate pyruvate transaminase), ALP (Alkaline phosphatase) and total bilirubin levels, as well as *in vivo* estimation of GSH (Glutathione) from liver tissue. The results were observed that the activity of SGOT, SGPT, ALP and total bilirubin was reduced in extract treated rats as compared to the disease control group rats[15].

- **Digamber R *et al.*, 2013** investigated *Pongamia pinnata* L., for its antifungal properties using different plant parts –leaves, bark, roots and seeds. The seeds of plants possessed highest antifungal activity followed by roots, bark and least activity was observed in the leaves. *Epidermophyton floccosum* was most susceptible to all the extracts closely followed by *Candida albicans* indicating the susceptibility to the components of the extracts. *Helminthosporium turcicum* was most resistant to all the extracts closely followed by *Alternaria solani* indicating the resistance to plant originated chemicals being familiar to such chemicals[16].

- **Selvaraju Kavipriya *et al.*, 2013** studied the antidiabetic activity of methanolic leaves extract of *Pongamia pinnata* L., with the dosage of 500mg/kg and 1g/kg was carried out in experimental animals. When the dosage level increased to 1g/kg, results revealed higher increment when compared to 500mg/kg. The methanolic leaf extract showed significant anti-hyperglycaemic activity at higher dose 1g/kg[17].

- **Divya Singh *et al.*, 2013** studied the anti-inflammatory and anti-arthritic activity of seed extract of *Pongamia pinnata* L., by *in vitro* model. *Pongamia pinnata* L., hydro alcoholic extract exhibited a concentration dependent inhibition of protein (albumin) denaturation. The stabilization of HRBC membrane showed a concentration dependent anti-inflammatory activity and the protection increased with increase in the concentration of the *Pongamia pinnata* L., hydroalcoholic extract[18].

- **SR Arote *et al.*, 2012** evaluated the hepatoprotective activity of the methanolic extract of *Pongamia pinnata* L., against paracetamol induced liver damage in rats. *In vitro* estimation of antioxidant activity was carried out using two different methods i.e.by hydroxyl radical scavenging activity and total reduction capability. The different doses (100, 200, 400 mg/kg) of the methanolic extract of *Pongamia pinnata* L., were screened for hepatoprotective and antioxidant activity. The AST, ALT, ALP values were significantly reduced in pre-treatment with methanolic extract of *Pongamia pinnata* L., group. It was also found that the methanolic extract of *Pongamia pinnata* L., showed dose dependant hydroxyl radical scavenging activity and total reduction capacity[19].

- **S. Aneela *et al.*, 2011** have reported the acute toxicity studies of crude seed extract of *Pongamia pinnata* L., in female Albino Wistar rats. The studies included the gross observation such as changes in body weight and food intake. The rats treated with dose of 2000mg/ kg body weight were safe[20].

- In a study by **Jimidi Bhaskar *et al.*, 2011**the methanol extracts of leaves of *Pongamia pinnata* L., produced significant (P<0.01) anti-pyretic activity. The 200mg/kg extracts has shown a good anti-pyretic effect (P<0.01) when compared to the control group. The results obtained indicate that the crude leaf extracts of *Pongamia pinnata* L., possess potent anti-pyretic activity[21].

- The anticonvulsant effect of 70% ethanol extract of *Pongamia pinnata* L., leaf against pentylene tetrazole induced convulsion (PTZ) in rats was evaluated by **Ashish manigauha *et al.*, 2010.** The ethanolic extract showed significant anticonvulsant activity by lowering the duration of extension phase (3.72± 0.65) when compared to control group (8.94 ± 0.42). Investigation of the anticonvulsant efficacy of 70% ethanol leaf extract of *Pongamia pinnata* L., using maximal electroshock-induced seizure (MES) in mice showed significant anticonvulsant activity characterised by of lowering the duration of extension phase (4.12 ± 0.67) when compared to control group (9.64 ± 0.41). These results indicate the potential anticonvulsant activity of the leaves of *Pongamia pinnata* L.,[22].

- The anti-hyperglycaemic and anti-lipidperoxidative activity of flowers of *Pongamia pinnata* L., were carried out by **Punitha *et al.*, 2006.** The oral administration of ethanolic extract of *Pongamia pinnata* L., flower shows significant anti-hyperglycaemic and anti-lipidperoxidative effect and also enhance antioxidant defense system in alloxan-induced diabetic rats[23].

- **Essa *et al.*, 2006** have evaluated the hepatoprotective effects of ethanolic extract o*f Pongamia pinnata* L., leaves in ammonium chloride induced hyperammonimia in rats. It was reported that the ethanolic extract offered protection against hepatotoxicity induced by Ammonium chloride by influencing the levels of lipid peroxidation products and liver marker enzymes. This activity is mediated by the antioxidant property of the leaf extract of *Pongamia pinnata* L.,[24].

- **Brijesh *et al.*, 2006** have reported the anti-microbial effect of crude leaf extract of *Pongamia pinnata* L., and have evaluated its effect on production and action of enterotoxins. Its extraction has no anti-bacterial, anti-giardial and anti-viral activities but reduces the production of cholera toxin and bacterial invasion to epithelial cells. This indicates that the extract of *Pongamia pinnata* L., has selective anti-diarrhoeal action with efficacy against cholera[25].

- Ethanolic extract of flowers of *Pongamia pinnata* L., was studied for its protective effect against cisplatin and gentamicin induced renal injury in rats **Shirwaikar *et al.*, 2004**. When the extract (300 & 600 mg/kg) was administered orally for 10 days following cisplatin (5 mg/kg, i.p.) on day 5, the flowers of *Pongamia pinnata* L., had a protective effect against cisplatin and gentamicin induced renal injury. The possible

mechanism of its protective effect against nephrotoxicity was attributed to its antioxidant activity[26].

- **Prabha *et al.*, 2003** have reported that the methanolic extract of *Pongamia pinnata* L., roots showed significant protection against aspirin and has a tendency to decrease acetic acid-induced ulcer after 10-days treatment. By augmentation of mucosal defensive factors like - mucin secretion, life span of mucosal cells, mucosal cell glycoproteins, cell proliferation and prevention of lipid peroxidation, the extract also shows ulcer protective effect[27].

- **Uddin *et al.*, 2003** investigated the antifilarial potential of the fruits and leaves extracts of *Pongamia pinnata* L., on cattle filarial parasite. In their investigation, the aqueous and alcohol extracts of fruits and the alcohol extract of leaves caused an inhibition of spontaneous movements of the whole worm and the nerve-muscle preparation of *Setar*ia. *cervi.* The concentration required to inhibit the movements of the whole worm preparation was 250µg/mL for aqueous, 120µg/mL for alcohol extract of fruits and 270µg/mL for alcohol extracts of the leaves. The concentrations of *Pongamia pinnata* L., extracts required to produce an equivalent effect on the nerve-muscle preparation were 25µg/ml, 5µg/ml and 20µg/ml[28].

- **Srinivasan *et al.*, 2003** evaluated the analgesic activity of the various root extracts of *Pongamia pinnata* L., The petroleum ether extract (PEE), n-Butanol extract (BE) and Ethanol extract (EE) of the roots of *Pongamia pinnata* L., showed significant analgesic effect in the tail flick test. The PEE and EE of the seeds also showed significant analgesic activity at doses higher than 100 mg/ kg[29].

- **Srinivasan *et al.*, 2001** evaluated the anti-inflammatory activity of ethanolic extract of *Pongamia pinnata* L., leaves (PPLE) in acute, subacute and chronic models of inflammation was assessed in rats. Oral administration of PPLE (300, 1000 mg/kg) exhibited significant anti-inflammatory activity in acute (carrageenin, histamine, 5-hydroxytryptamine and prostaglandin E_2-induced hind paw edema), subcute (kaolin-carrageenin and formaldehyde-induced hind paw edema) and chronic (cotton pellet granuloma) models of inflammation. PLE did not show any sign of toxicity and mortality up to a dose level of 10.125 g/kg, p.o in mice. Both acute as well as chronic administration of PLE (100, 300 and 1000 mg/kg, p.o) did not produce any gastric lesion in rats. These results indicate that PLE possesses significant anti-inflammatory activity without ulcerogenic activity suggesting its potential as an anti-inflammatory agent for use in the treatment of various inflammatory diseases[30].

- **Simonsen *et al.*, 2001** in their study have reported that the *Pongamia pinnata* L., shows anti-plasmodial activity against *Plasmodium falcipar*[31].

- The antibacterial activity of various extracts of *Pongamia pinnata* L., seeds was evaluated by **Dayanand *et al.*, 2013.** The methanol extracts of *Pongamia pinnata* L., *L* (PPM) showed higher antibacterial activity than ethanol extracts of *Pongamia pinnata* L., (PPE). *Pongamia pinnata* L., *L* has good bactericidal activity against the selected Hospitalized pathogens and the maximum activity was evinced on *Pseudomonas aeruginosa*, with a zone of inhibition 20mm by methanol extract and 18.5mm on *Pseudomonas aeruginosa* in ethanol extract in comparison to Ceftazidime[32].

- **Singh R K *et al.*, 1997** have reported that the petroleum ether extract (PEE) of the roots enhanced pentobarbitone sleeping time, probably due to CNS depression. The PEE of the seed of *Pongomia pinnata* L., was further tested for nootropic activity in an experimental model of Alzheimer's disease (created by ibotenic acid induced lesioning of nuclear basali magnocellularis). It reversed both the cognitive deficits and the reduction in cholinergic markers after 2 weeks of treatment. Reversal of perturbed cholinergic function was considered as the possible mechanism[33].

2.2. PHYTOCHEMICAL STUDIES

- **Prashanth G.K *et al.*, 2014** have reported the phytochemical screening of the aqueous and ethanolic extracts of the leaves of *Pongamia pinnata* L., revealed the presence of alkaloids, carbohydrates, reducing sugars etc. GC-MS analysis of the ethanolic extract indicated the presence of many constituents in the leaves of *Pongamia pinnata* L., such as Alkaloids, Glycoside, Saponins, Tannins and phenolic compounds[34].

- **Vismaya *et al.*, 2010** have reported the Karanja (*Pongamia pinnata* L.,) seed oil contains karanjin, a bioactive molecule with important biological attributes. They subjected the seed oil to liquid–liquid extraction with methanol. The extract was further purified by chromatography on alumina followed by crystallization to afford karanjin, whose purity was ascertained by HPLC. They obtained the recovery of karanjin as 20% with >95% purity. The structure of the compound was elucidated by MS and NMR spectral analysis[35].

- Isolation and characterization of five structurally unusual flavonoids pongamones A–E, along with 16 known flavonoid metabolites were carried out by from the stem of *Pongamia pinnata* L., by **Li *et al* 2006**. Their structures were determined on the basis of spectroscopic analyses and by comparison of their spectroscopic data with those of related compounds reported in the literature[36].

- **Yadav *et al.,* 2004** isolated four new furanoflavonoids, pongapinnol A–D and a new coumestan, pongacoumestan along with thirteen known compounds from the fruits of *Pongamia pinnata* L., They elucidated the structures of isolated compounds on the basis of spectroscopic data interpretation[37].

- **Kalidhar *et al.,* 2003** investigated the chemical constituents of the roots of *Pongamia pinnata* L., Four compounds, karanjin, pongachromene, pongapin and demethoxykanugin were characterized from the methanolic extract of the roots[38].

- Six compounds (two sterols, three sterol derivatives and one disaccharide) together with eight fatty acids (three saturated and five unsaturated) have been isolated from the seeds of *Pongamia pinnata* L., by **Shameel S *et al*., 1996**. The metabolites, beta-sitosteryl acetate and galactoside, stigma sterol, its galactoside and sucrose are being reported from this plant. The saturated and unsaturated fatty acids (two monoenoic, one dienoic and two trienoic) were present in exactly the same amount. Oleic acid occurred in highest amount (44.24%), stearic (29.64%) and palmitic (18.58%). Hiragonic and octadecatrienoic acids were present in trace amounts (0.88%). Karangin, pongamol, pongagalabrone and pongapin, pinnatin and kanjone have been isolated and characterized from seeds. Immature seeds contain a flavone derivative

pongol. The other flavonoid isolated from the seeds includes glybanchalcone, isopongachromene[39].

2.3. LIST OF REVIEW ON THE MODELS USED FOR HEPATOPROTECTIVE EVALUATION

Various models used to investigate the heptoprotective activity are,

- CCl_4 induced hepatotoxicity[40]
- Ethanol induced hepatotoxicity[41]
- Paracetamol induced hepatotoxicity[15]
- Lead acetate induced hepatotoxicity[42]

CCl_4 induced hepatotoxicity is one of most commonly used models for the evaluation hepatoprotective activity. The hepatoprotective activity of various plants was carried out by utilising CCl_4 model as an inducing method. Some of the plants that have been evaluated for hepatoprotecive activity using CCl_4 model are *Viburnum punctatum*[43], *Eurycoma longifolia*[44], *Vitis vinifera*[45], *Cissampelos pareira*[46], *Prunnus domestica*[47], *Parmelia perlata*[48], *Mallotus mull-Arg*[49], *Mimosa pudica*[50], *Solanum trilobatum*[51].

3. PLANT PROFILE

Pongamia pinnata (Linn.,) belongs to the family Papilionaceae.

SYNONYMS

Derris indica(Lam.,)Bennett.

Millettia novo - guineensis Kane.

Pongamia glabra Vent.

Pongamia pinnata Merr

BOTANICAL CLASSIFICATION

Kingdom	:	Plantae
Subkingdom	:	Tracheobionta
Superdivision	:	Spermatophyta
Division	:	Magnoliophyta
Class	:	Magnoliopsida
Subclass	:	Rosidae
Order	:	Fabales
Family	:	Leguminoseae
Genus	:	*Pongamia*
Species	:	*Pinnata*

BOTANICAL NAME

*Pongamia pinnata (*L.,*)* pierre

VERNACULAR NAMES[52]

Hindi, Beng., Mar. and Guj	:	Karanj, Karanja
Sanskrit	:	Naktamala
English	:	Indian beech
Telugu	:	Pungu, Gaanuga

Tamil	:	Ponga, Pongam
Malayalam	:	Pungu, Punnu
Oriza	:	Koranjo
Punjab	:	Sukhehein, Karanj, Paphri
Assam	:	Karchuw

Figure. 4

A

B

C

D

Figure A **:** *Pongamia pinnata* L., whole plant

Figure B : Leaves

Figure C : Flower

Figure D : Seeds

GEOGRAPHICAL DISTRIBUTION

It is widely distributed throughout tropical Asia and the Seychelles Islands, South Eastern Asia, Australia, India and locally distributed throughout the State of Maharashtra (India) along the banks of rivers, very common near the sea-coast in tidal and beach-forests in Konkan along Deccan rivers[53].

BOTANICAL DESCRIPTION

Plant type

Medium - sized, evergreen, perennial and deciduous tree

Height - 35 to 40 feet

Growth rate - Fast

Texure - Medium

Growing requirements

Light requirement - Tree grows in full sun.

Soil tolerance - Clay, loam, sandy, slightly alkaline, acidic, well-drained.

Macroscopy

Leaf - Alternate, odd pinnately compound, evergreen, hairless.

Flower - Lavender, pink; white, 2- 4 together, short-stalked, pea shaped, 15-18mm long.

Pods - 3-6cm long and 2-3cm wide, smooth, brown, thick-walled, hard, indehiscent.

Seed - Compressed ovoid or elliptical, bean-like, 10-15cm long, dark brown, oily.

Root - Tap root is thick and long, lateral roots are numerous and well developed.

Bark - Thin gray to grayish brown and yellow on the inside.

PHYTOCHEMICAL CONSTITUENTS

Phytochemical investigation of *Pongamia pinnata* L., indicated the presence of abundant prenylated flavonoids such as furanoflavones, furanoflavonols, chromenoflavones, furanochalcones and pyranochalcones.

Karanja *(Pongamia pinnata* L.,*)* seed oil contains karanjin a bioactive molecule with important biological attributes.

Pongamia pinnata L., contain 30 to 40% pongam oil and also called pongomol or Hongay oil.

The total saturated and unsaturated fatty acid composition was 20.5% and 79.4% respectively. The major mono unsaturated fatty acid was oleic acid 46% whereas linoleic acid 27.1% and Linolenic acid 6.3% constitutes the total polyunsaturated fatty acid. Low molecular weight fatty acids such as lauric and capric acids occure in very small amount of about 0.1% each.

Table - 1

ECONOMIC AND MEDICINAL IMPORTANCE OF *PONGAMIA PINNATA* (L.,) [53,54]

Leaf	**Economic value**	Used as cattle fodder. Used in stored grains to repel insects. Used as manure for rice and sugarcane fields
	Medicinal value	Juice of leaves is used for cold, cough, diarrhea, dyspepsia, flatulence, gonorrhea, leprosy. Leaves are antihelminthitic, digestive and laxative used for inflammations, piles, hepatoprotective and wounds. As an infusion to relieve rheumatism. As an extract to treat itches and herpes.
Flower	**Economic value**	Good source of pollen for honey bees. Flowers are edible.
	Medicinal value	Useful to quench dipsia in diabetes for alleviating vata kapha and for bleeding piles
Root	**Economic value**	Root is used as fish poison
	Medicinal value	Juice of roots with coconut milk and lime water used for treatment of gonorrhea. Used for cleaning gums, teeth and ulcers. Roots are bitter anti-helmintic and used in vaginal and skin diseases.

		Juice of the root is used for cleansing foul ulcers and closing fistulous sores.
Fruit	**Economic value**	Fruits are edible
	Medicinal value	Fruits used for abdominal tumors. Useful in ailments of female genital tract, leprosy, tumour, piles, ulcers and upward moving of the wind in the abdomen.
Seed	**Economic value**	After oil extraction the marc has been used as "green manure" as it is rich in protein and nitrogen. Used as insecticides.
	Medicinal value	Used for keloid tumors. Used in hypertension, skin ailments and rheumatic arthritis. Seed powder valued as a febrifuge, tonic and in bronchitis and whooping cough. Useful in inflammations, pectoral diseases, chronic fevers, hemorrhoids and anemia.
Stem	**Economic value**	Used for stove top fules. Used as poles and carving Ash of wood used for drying Agriculture implements, tool handles and combs.
	Medicinal value	Aqueous extracts of stem bark exhibit significant CNS sedative and antipyretic activity.
	Economic value	Used as fuel for cooking and lamps, as a lubricant, water-paint binder, pesticide and in soap-making, candles and tanning industries.

Oil		Used as lipids for commercial processes. Used in cosmetics.
	Medicinal value	Oil is styptic, anthelmintic and useful in leprosy, piles, ulcers, chronic fever and in liver pain. Useful in rheumatism arthritis, scabies, whooping cough. Mixture of oil and zinc oxide used for eczema.
Bark	**Economic value**	String and rope can be made from the bark fiber. Used for paper pulp
	Medicinal value	For bleeding piles, for beriberi, reduces swelling of the spleen. Useful in mental disorder, cough and cold.

4. AIM AND OBJECTIVE

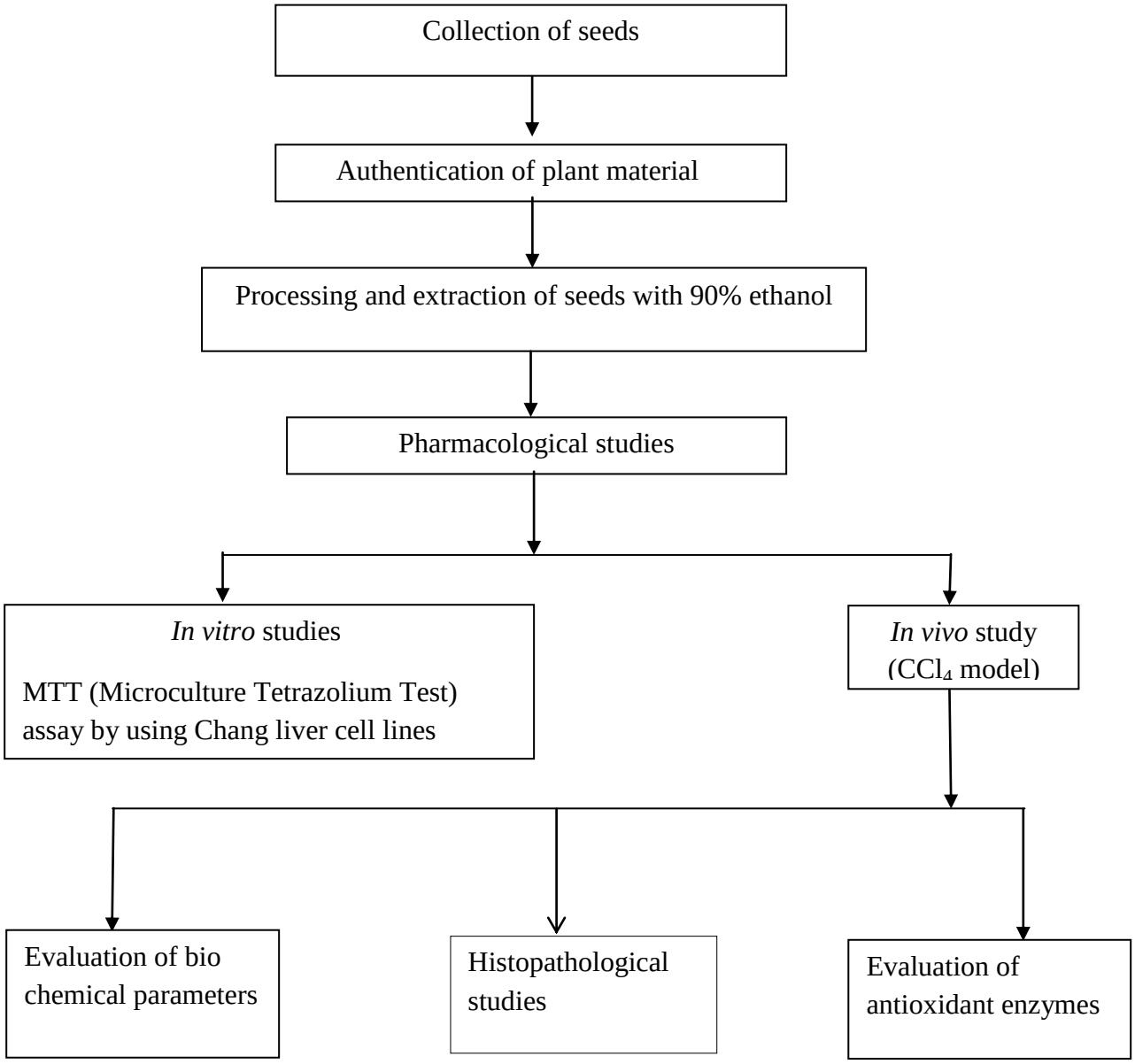
From the literature review it has been found that

- *Pongamia pinnata* L., has been reported to possess antioxidant activity and antioxidants are known to be associated with hepatoprotective activity. Plants containing flavonoids are known to exihibit the hepatoprotective activity.
- In this plant, the leaves and stem bark of *Pongamia pinnata* L., have been shown to possess hepatoprotective activity. No work has so far been carried out on the seeds of *Pongamia pinnata* L.,

Hence, the aim and objective of the present study is

- To evaluate the *in vitro* hepatoprotective activity of the seeds of *Pongamia pinnata* L., by MTT assay using normal Chang liver cell line.
- To evaluate the *in vivo* hepatoprotective activity of the seeds of *Pongamia pinnata* L., by using Carbon tetrachloride induced hepatotoxicity model in Wistar rats.

5. PLAN OF WORK



6. MATERIALS AND METHODS

6.1. Plant collection and identification[55-57]

Dried seeds of *Pongamia pinnata* L., were collected from the forest around Kutralam hills, Tirunelveli District, Tamilnadu (India) in the month of August 2015. The plant was authenticated by Prof. V. Chelladurai, Research Officer- Botany (Scientist - C) (Retd), Central Council for Research in Ayurveda & Siddha, Govt. of India.

6.2. Preparation of plant extract

The freshly collected seeds of this plant was chopped, shade dried. The dried material was powdered and passed through a 10-mesh sieve. The powder was then extracted with ethanol (90%) using a Soxhlet extractor. In this process, the vapour from the solvent is carried to the condenser, where it condenses liquid returns to the flask for continuous extraction.

Procedure

- 50g of dried powdered seeds was weighed and transferred into thimble of the Soxhlet apparatus for packing.
- While packing, the content was wetted with 90% ethanol (the solvent used) and then poured until the siphon tube was filled.
- A piece of porcelain was added into the round bottom flask to avoid bumping effect.

The extracts were concentrated by using a rotary evaporator. The extract was then subjected to *in vitro* and *in vivo* hepatoprotective evaluation.

6.3. *IN VITRO* STUDIES

6.3.1. *IN VITRO* HEPATOPROTECTIVE ACTIVITY BY MTT ASSAY USING CHANG LIVER CELL LINE[58]

The ethanolic extract of seeds of *Pongamia pinnata* L., was subjected to evaluation of *in vitro* hepatoprotective activity.

Reagents

- MEM (Minimal Essential Media) purchased from Hi Media Laboratories, Mumbai
- FBS (Fetal Bovine Serum) purchased from Cistron laboratories
- Trypsin, MTT [3-(4, 5- Dimethyl thiazol -2-yl) - 5- diphenyl tetrazolium bromide] was purchased from Sisco Research Laboratory Chemicals, Mumbai.
- DMSO (Dimethyl sulfoxide) was purchased from Sisco Research Laboratory Chemicals, Mumbai.

Cell line and culture

- Normal Chang liver cell lines were obtained from National Centre for Cell Sciences (NCCS), Pune. The cells were maintained in Minimal Essential Medium supplemented with 10% FBS, Penicillin (100 U/ml) and Streptomycin (100 µg/ml) in a humidified atmosphere at 37ºC.
- Maintenance of cultures was passaged weekly and the culture medium was changed twice a week.

MTT ASSAY

Principle

MTT assay is a standard colorimetric assay used for measuring the viability of cells. It can be used indirectly to evaluate the cytotoxicity of the sample under study.

The assay is based on conversion of the MTT [3-(4, 5 – dimethyl thiozol – 2-yl)-2, 5-diphenyl tetrazolium bromide], a yellow tetrazole to a purple coloured formazan crystal by

the active mitochondrial reductase (or cellular reductase) present in the viable cells. The colour solution can be quantified by measuring absorbance, the purple colour thus formed is directly proportional to the number of viable cells present.

Procedure

Cytotoxicity evaluation by tetrazolium (MTT) assay

- The Chang liver monolayer cells were detached with Trypsin-ethylene diamine tetra acetic acid (EDTA) to make single cell suspension and the viable cells were counted using a haemocytometer and diluted with medium along with 5% Fetal Bovine Serum (FBS) to give final density of 1×10^5 cells/ml.
- Cells (1×10^5 cells/ml) were plated in 5ml of medium/well in 96 well plates (Coster Coring, Rochester, NY).
- After 48 hours incubation, the cell reaches the confluence.
- Then, cells were incubated with different concentrations of Silymarin, EPPS for 24 hours at 37ºC.
- After removal of the sample solution and washing with phosphate-buffered saline (pH 7.4), 200µl of (5mg/ml) of 3-(4, 5 – dimethyl thiozol - 2-yl)-2, 5- diphenyl tetrazolium bromide cells (MTT) phosphate-buffered saline solution was added each well.
- After 4 hour incubation, 0.04M HCl/isopropanol were added.
- Viability of cells was determined by measuring the absorbance at 570nm using UV spectrophotometer and wells not containing sample were treated as blank.
- Measurements were performed and the concentration required for a 50% inhibition of viability (IC_{50}) was determined graphically.
- Triplicate was maintained for all concentrations.

- The effect of the samples on the proliferation of Chang Liver cells was expressed as the % cell viability, using the formula

$$\% \text{ cell viability} = \frac{\text{OD of treated cells}}{\text{OD of control cells}} \times 100$$

6.3.1.2. *IN VITRO* HEPATOPROTECTIVE ACTIVITY OF SILYMARIN AND EEPPS AGAINST CCl_4 INDUCED TOXICITY BY MTT ASSAY USING CHANG LIVER CELL LINE

Since the extract was found to be as safe Silymarin and the CTC_{50} value of both EEPPS, Silymarin were approximately 100µg/ml, the hepatoprotective activity was determined at various concentration with a maximum of 100 µg/ml.

Reagents

- MEM (Minimal Essential Media) purchased from Hi Media Laboratories, Mumbai
- FBS (Fetal Bovine Serum) purchased from Cistron laboratories
- Trypsin, MTT [3-(4, 5- Dimethyl thiazol -2-yl) - 5- diphenyl tetrazolium bromide] was purchased from Sisco Research Laboratory Chemicals, Mumbai.
- DMSO (Dimethyl sulfoxide) was purchased from Sisco Research Laboratory Chemicals, Mumbai.

Cell line and culture

- Normal Chang liver cell lines were obtained from National Centre for Cell Sciences (NCCS), Pune. The cells were maintained in Minimal Essential Medium supplemented with 10% FBS, Penicillin (100 U/ml) and Streptomycin (100 µg/ml) in a humidified atmosphere at 37ºC.Maintenance of cultures was passaged weekly and the culture medium was changed twice a week.

MTT ASSAY

Principle

MTT assay is a standard colorimetric assay used for measuring the viability of cells. It can be used indirectly to evaluate the cytotoxicity of the sample under study.

The assay is based on conversion of the MTT [3-(4, 5 – dimethyl thiozol - 2-yl)-2, 5-diphenyl tetrazolium bromide], a yellow tetrazole to a purple coloured formazan crystal by the active mitochondrial reductase (or cellular reductase) present in the viable cells. The colour solution can be quantified by measuring absorbance, the purple colour thus formed is directly proportional to the number of viable cells present.

Procedure

The Chang liver monolayer cells were detached with trypsin-ethylene diamine tetra-acetic acid (EDTA) to make single cell suspensions and viable cells were counted using Haemocytometer and diluted with medium along with 5% FBS to give final density of 1×10^5 cells/ml.

- Cells (1×10^5/well) were plated in 5ml of medium/well in 96 well plates (Coster Coring, Rochester, NY).
- After 48 hours incubation, the cell reaches the confluence.
- Then, cells were challenged with Hepatotoxicant (CCl_4) 125µg/ml and different concentration (100, 50, 25, 10µg/ml) of extract and the Standard drug (Silymarin) were added. The cells were incubated for 24-48 hours at 37ºC.
- After removal of the sample solution and washing with phosphate-buffered saline (pH 7.4), 200µl of (5mg/ml) of 3- (4, 5 - dimethyl thiozol -2-yl)-2, 5- diphenyl tetrazolium bromide cells (MTT) phosphate-buffered saline solution was added each well.
- After 4 hour incubation, 0.04M HCl/isopropanol were added.

- Viability of cells was determined by measuring the absorbance at 570nm using UV spectrophotometer and wells not containing sample were treated as blank.
- Measurements were performed and the concentration required for a 50% inhibition of viability (IC_{50}) was determined graphically.
- Triplicate was maintained for all concentrations.
- The effect of the samples on the proliferation of Chang Liver cells was expressed as the % cell viability, using the formula

$$\textbf{\% cell viability} = \frac{\textbf{OD of treated cells}}{\textbf{OD of control cells}} \times \textbf{100}$$

6.4. *IN VIVO* STUDIES

Experimental Animals

The present study was conducted after obtaining approval from the **I**nsitutional **A**nimal **E**thics **C**ommittee, Madras Medical College, Chennai-03 and this protocol met the requirements of national guidelines of CPCSEA **(PROPOSAL NO: 03/243/CPCSEA)** The Wistar rats (150-200g) used for this study were procured from, **I**nsitutional **A**nimal **E**thics **C**ommittee, Madras Medical College, Chennai-03, India.

Quarantine and Acclimatization

Quarantine is the separation of newly received animals from those already in the facility until the health and possibly the microbial status of the newly received animals have been determined. The newly procured Wistar albino rats were quarantined for the period of one week to minimize the chance of introduction of pathogens into established animals and allowed to develop the physiological and nutritional stabilisation before their use.

Housing

The animals were housed in well ventilated animal house which was maintained at a constant temperature and relative humidity of 55 – 65%. The animals were housed in spacious polypropylene cages and paddy husk was utilized as bedding material.

Diet and water

The animals were maintained on standard pellet diet and purified water. The animals were provided with food and water *ad libitum* expect during fasting. The bedding material was changed frequently.

Drug administration

Drug was administered by oral gavage using oral feeding tube fixed to a syringe needle to administer the required quantity of the drug.

Animal identification

All animal cages used in the study had a proper identification i.e. labels. Each animal in the cage was marked either on head or body or tail with picric acid for their appropriate identification.

6.4.1. *IN VIVO* HEPATPROTECTIVE EVALUATION OF EEPPS WITH CCl_4 MODEL OF HEPATOTOXICITY[41,46,59]

The ethanolic extract of seeds of *Pongamia pinnata* L., was subjected to evaluation of *in vivo* hepatoprotective activity.

Drugs and chemicals

- Carbon tetrachloride
- Silymarin
- β - cyclo dextrin (BCD)

Preparation of drug solutions

Carbon tetrachloride was dissolved in olive oil 1:1

Ethanol extract was dissolved in 1% BCD

Standard drug (Silymarin) was dissolved in distilled water.

Experimental method

Carbon tetrachloride induced hepatotoxicity in wistar albino rats was used as a model for screening for hepatoprotective activity.

6.4.1.1. Acute toxicity study

Acute toxicity studies have been already done and it was found to be safe upto 2000mg/kg p.o. Hence, the doses selected for this study were 200mg/kg and 400mg/kg p.o.[23]

HEPATOPROTECTIVE EVALUATION

Rats were divided into five groups consisting of 6 animals each.

GROUP 1 : Received a single oral dose of BCD (1ml of 1%, w/v)

GROUP 2 : The animals were injected a mixture of carbon tetrachloride in olive oil in a dose of 1ml/kg body weight, i.p, once every 72 hours for 14 days and received daily a single oral dose of BCD (1ml of 1%, w/v)

GROUP 3 : The animals received daily oral dose of standard drug Silymarin 25mg/kg for 14 days. The mixture of carbon tetrachloride in olive oil (1ml/kg body weight, i.p, once every 72 hours for 14 days and received daily a single oral dose of BCD (1ml of 1%, w/v)

GROUP 4 : The animals received daily oral dose of 200mg/kg body weight of ethanolic extract of *Pongamia pinnata* L., seeds (EEPPS) in BCD.

GROUP 5 : The animals received daily oral dose of 400mg/kg body weight of ethanolic extract of *Pongamia pinnata* L., seeds (EEPPS) in BCD.

S. NO	GROUP (n=6)	TREATMENT	TREATMENT SCHEDULE
1	I	Normal control	1ml of 1% BCD p.o for 14 days
2	II	Disease control	1ml/kg CCl_4 in olive oil (1:1), i.p, once in every 72h for 14 days
3	III	Standard control	1ml/kg CCl_4 in olive oil (1:1), i.p, once every 72 h for 14 days + 25mg/kg p.o of Silymarin, daily for 14 days
4	IV	Test group I	1ml/kg CCl_4 in olive oil (1:1), i.p, once every 72 h for 14 days + 200mg/kg of EEPPS p.o, daily for 14 days
5	V	Test group II	1ml/kg CCl_4 in olive oil (1:1), i.p, once every 72h for 14 days + 400mg/kg EEPPS p.o, daily for 14 days

For all rats, body weight was measured before and after the induction of hepatotoxicity (1^{st} and 15^{th} day). On the 15^{th} day, blood samples were collected by retro orbital puncture for estimation of biochemical parameters. The animals were sacrificed by cervical dislocation method. The liver was removed rinced in ice cold saline and stored in 10% formalin solution. A part of liver was homogenised with Phosphate buffer, pH 7.4 using a Teflon homogenizer in ice-cold condition. The homogenate was centrifuged at 5000 rpm for 10min. The supernatants solution was taken up for the evaluation of Lipid peroxidation (LPO),

Superoxide dismutase (SOD) levels. The other part of liver was subjected to histopathological study.

6. 5. BIOCHEMICAL PARAMETERS

The blood samples were collected and allowed to clot and centrifuged at 2000 rpm for 15-20 minutes using REMI (412 LAG) cooling centrifuge. The serum was kept -80° C until analysed. Levels of Total protein, Albumin, Total Bilirubin, Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), Serum alkaline phosphatase (ALP) were determined with an Automated Analyser (Hitachi 911, Japan).

6.5.1. Estimation of Aspartate Aminotransferase[60] (AST/SGOT)

Aspartate aminotransferase (AST) also referred to as Serum glutamate oxaloacetate transferase (SGOT), is an enzyme involved in amino acids metabolism. AST is widely distributed in liver, RBC, heart, pancreas and kidney. A high level of AST in blood is observed in severe liver disease, kidney disease and lung disease.

Principle

$$\alpha\text{-Ketoglutarate} + \text{L-Aspartate} \xrightarrow{\text{SGOT}} \text{L-Glutamate} + \text{Oxaloacetate}$$

$$\text{Oxaloacetate} + \text{NADH} + H^+ \xrightarrow{\text{MDH}} \text{L-Malate} + NAD^+$$

The rate of NADH consumption is measured photometrically and is directly proportional to the AST concentration in the sample.

Reagents

- L – Aspartate 200mmol/l
- Malate dehydrogenase 200mmol/l
- ∝ - Ketoglutarate 35mmol/l
- NaOH 1.05mmol/l

Procedure

800µl of L - Aspartate & Malate dehydrogenase and 200µl of ∝ - Ketoglutarate are mixed together and incubated at 37ºC for 2 minutes and 100 µl of sample is added. The change in absorbance is measured at 340nm.

Calculation

AST = Abs/ min × 1764(Factor)

6.5.2. Estimation of Alanine Aminotransferase[61] (ALT/SGPT)

Alanine aminotransferase/ Serum Glutamate Pyruvate Transferase is an enzyme which is involved in amino acids metabolism.

Principle

∝ - Ketoglutarate + L – Alanine $\xrightarrow{\text{SGPT}}$ **L – Glutamate + Pyruvate**

Pyruvate + NADH + H^+ $\xrightarrow{\text{LDH}}$ **L – Lactate + NAD^+**

The rate of NADH consumption is measured photometrically and is directly proportional to the SGPT concentration in the sample.

Reagents

- L – Alanine 200mmol/l
- Lactate Dehydrogenase 1500mmol/l
- ∝ - Ketoglutarate 35mmol/l
- NADH 1.05mmol/l
- Tris buffer 80mmol/l pH 7.5

Procedure

800µl of L – Alanine and Lactate dehydrogenase and 200µl of ∝ - Ketoglutarate are mixed together and incubated at 37ºC for 2minutes and 100µl of sample is added. The change in absorbance is measured at 340nm.

Calculation

ALT = ΔAbs/min × 1764(Factor)

6.5.3. Estimation of Alkaline Phosphatase (ALP)

Principle

When the enzymes incubated with p-nitro phenyl phosphate and Tris buffer (pH 9.6) in alkaline condition inorganic phosphate and p-nitro phenol are formed by the catalytic action of alkaline phosphatase. Amount of p-nitro phenol liberated by the enzyme is measured at 420nm.

2-amino-methyl-1-propanol+p-nitro phenyl phosphate + H_2O

↓

4-nitro phenol+ 2-amino-2-methyl-1-propanol phosphate

Requirements

- P-nitro phenyl phosphate (10mM)
- Tris-HCl pH 9.6 (80mM)
- NaOH (0.1N)

Procedure

1ml of p-nitro phenyl phosphate and 1.5ml of buffer were added with 100µl of homogenate. The mixture was incubated at 37ºC for 30mins. Then the reaction was stopped by addition of 0.1 N NaOH. The absorbance of liberated p-nitro phenol was measured at 420nm.

Calculation

ALP U/I = ΔA/ 2764

6.5.4. Estimation of Total Bilirubin (TB)

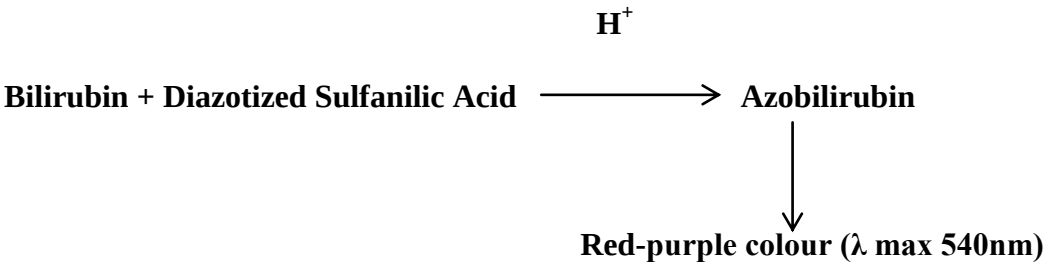
Principle

Method of Malloy and Evelyn used to estimate serum bilirubin level.

Direct : (Conjucated) Bilirubin couples with diazotized Sulfanilic acid, forming Azobilirubin, a red-purple coloured product in acidic medium.

Indirect : (Unconjucated) Bilirubin is diazotized only in the presence of its dissolving solvent (Methanol). Indirect fraction and thus represents Total bilirubin concentration. The difference of Total and Direct Bilirubin gives Indirect (Unconjucated) Bilirubin.

The intensity of red-purple colour so developed above is measured calorimetrically and it id proportional to the concentration of the appropriate fraction of Bilirubin. This reaction can be represented as:



Reagents

Diazo-A, Diazo-B and Diazo blank

Methanol

Artificial standard (100mg bilirubin)

Diazo reagent: Just before use, mix 1.0ml of Diazo-A with 0.030ml of Diazo-B

Procedure

0.1ml of serum, 0.25 ml of diazo reagent and methanol 1.25ml were mixed well and kept in dark at room temperature for 30mins and read the optical density (O.D) at 540nm against distilled water on a colorimeter with a yellow green filter.

Calculation

$$\textbf{Total Bilirubin} = \frac{\textbf{O.D.}_{\textbf{T1}} - \textbf{O.D.}_{\textbf{T2}}}{\textbf{O.D. Standard}} \times \textbf{10}$$

6.5.5. Estimation of Total Protein (TP) [62] (Biuret Method)

The serum content of the soluble proteins, those circulating in extracellular and intracellular fluids, has been used as a marker to aid in clinical diagnosis. Serum total protein including albumin is mainly involved in the maintenance of osmotic pressure of plasma and is used to transport many substances including macromolecules.

Principle

In the biuret reaction, a chelate is formed between the Cu^{2+} ion and the peptide bonds of the proteins in alkaline solutions to form a violet coloured complex whose absorbance is measured photometrically at 540nm. The intensity of the colour produced is proportional to the concentration of the protein in the sample.

$$\textbf{Cu}^{2+} + \textbf{Serum protein} \longrightarrow \textbf{Copper-protein complex}$$

(Violet coloured)

Reagents

- Total protein reagent (Biuret reagent)
- Protein standard (Standard prepared from bovine serum albumin)
- Serum sample from animals

Procedure

1ml of biuret reagent which is stored under 2-8ºC and 10µl of serum sample/standard are mixed well and incubated for 5mins. The intensity of the colour is measured at 540nm.

Calculation

$$\text{Total protein in gm/ml} = \frac{\text{Absorbance of test}}{\text{Absorbance of standard}}$$

6.5.6 Estimation of Albumin (ALB)

Principle

Bromocresol-Green (BCG) method

When albumin binds with bromocresol green in a suitable buffer, pH 4.15-4.25, an intense blue coloured albumin-BCG complex is formed

Albumin + BCG (Yellow) $\longrightarrow$ Albumin-BCG complex (blue)

Reagents

Bromocresol green reagent

Serum sample from animals

Procedure

1ml of bromocresol reagent, 1.5ml of buffer and 0.2ml serum were mixed and kept at room temperature for 10mins. The absorbance was measured at 630nm.

6.6 ESTIMATION OF LIPID PEROXIDATION AND SUPEROXIDE DISMUTASE LEVELS

6.6.1. Estimation of Lipid Peroxidation (LPO) [63]

The extent of lipid peroxidation was estimated as evidenced by the formation of Thiobarbituric acid reactive substance (TBARS). The pink colored chromogen formed by the reaction of 2-thiobarbituric acid (TBA) with breakdown products of lipid peroxidation was read at 532 nm.

Reagents

1. 8.1% sodium dodecyl sulphate (SDS)
2. 20 % v/v acetic acid.
3. 0.5% v/v TBA
4. n-butanol and pyridine (15:1 v/v)
5. Stock malondialdehyde solution

   1,1,3,3 - tetramethoxy propone (184 µg/ml)

Procedure

To 0.2 ml of liver tissue homogenate, 0.2 ml of 8.1 % SDS, 1.5 ml of 20 % acetic acid, solution (adjusted to pH 2 to 3.5 with NaOH) and 1.5 ml of 0.8 % aqueous solution of TBA were added. The mixture was made up to 4.0 ml with distilled water and then heated in a water bath at 95°C for 60 minutes. After cooling with tap water, 1.0 ml of distilled water and 5.0 ml of a mixture of n-butanol and pyridine were added and shaken vigorously. After centrifugation at 4000 g for 10 minutes, the organic layer was removed and its absorbance at 532nm was measured.

Values were expressed as n moles/100 mg protein.

6.6.2. Estimation of Superoxide Dismutase (SOD) [64]

The assay is based on the inhibition of the formation of NADH-phenazine methosulphate-nitroblue tetrazolium formazon. The reaction was initiated by the addition of NADH. After incubation for 90 sec, addition of glacial acetic acid stops the reaction. The colour developed at the end of the reaction was extracted into n-butanol layer and measured in a spectrophotometer at 520 nm.

Reagents

1. Sodium pyrophosphate buffer: 0.025 M, pH. 8.3
2. Absolute ethanol
3. Chloroform
4. n-butanol
5. Phenazine methosulphate (PMS): 186 μmol
6. Nitroblue tetrazolium (NBT) : 300 μmol
7. NADH : 780 μmol

Procedure

0.5 ml of tissue homogenate was diluted to 1.0 ml with water followed by addition of 2.5 ml of ethanol and 1.5 ml of chloroform (chilled reagents were added). This mixture was shaken for 90 sec at 4 °C and then centrifuged. The enzyme activity in the supernatant was determined as follows. The assay mixture contained 1.2 ml of sodium pyrophosphate buffer, 0.1 ml of phenazine methosulphate and 0.3 ml of nitroblue tetrazolium and appropriately diluted enzyme preparation in a total volume of 3 ml. The reaction was started by the addition of 0.2 ml NADH. After incubation at 30 °C for 90 sec, the reaction was stopped by the addition of 1 ml glacial acetic acid. The reaction mixture was stirred vigorously and shaken

with 4 ml n-butanol. The mixture was allowed to stand for 10 min, centrifuged and n-butanol layer was separated. The colour density of the chromogen in n-butanol was measured at 510 nm. A system devoid of enzyme served as control. The enzyme concentration required to inhibit the chromogen produced by 50% in one minute under standard conditions was taken as one unit.

SOD activity was expressed as Unit/min/mg of protein

6.7. HISTOPATHOLOGICAL STUDIES[65]

The liver from the animals was rinsed in ice cold 0.9% saline and was fixed in 10% formalin embedded in paraffin and cut into 5µm thick section using a microtome. Sections were mounted on glass slides using standard techniques. The sections were stained with Haematoxylin - Eosin and were examined under a microscope using 100 x magnifications and photographed under a light microscope equipped for photography (Olympus CK 40).

STATISTICAL ANALYSIS

All the values were expressed as mean ± SD. The data was statistically analysed by one way ANOVA followed by Dunnet's test. One way analysis of variance (ANOVA) was used to correlate the statistical difference between the variables. $P<0.05$ was considered to be significant.

7. RESULTS

6.3. *IN VITRO* STUDIES

The ethanolic extract of *Pongamia pinnta* L., seed was prepared by Soxhlet extraction. The extract was greenish brown in colour and it was semi solid in nature.

6.3.1. Cytotoxicity evaluation by MTT assay

The cytotoxicity studies were carried out for the standard drug Silymarin and ethanolic extract of *Pongamia pinnata* L., seeds (EEPPS) on the Chang liver cell line using different concentration range (50, 100, 200, 300,400, 500µg/ml) and its CTC_{50} values was determined. **(Table 2and Figure. 5)**

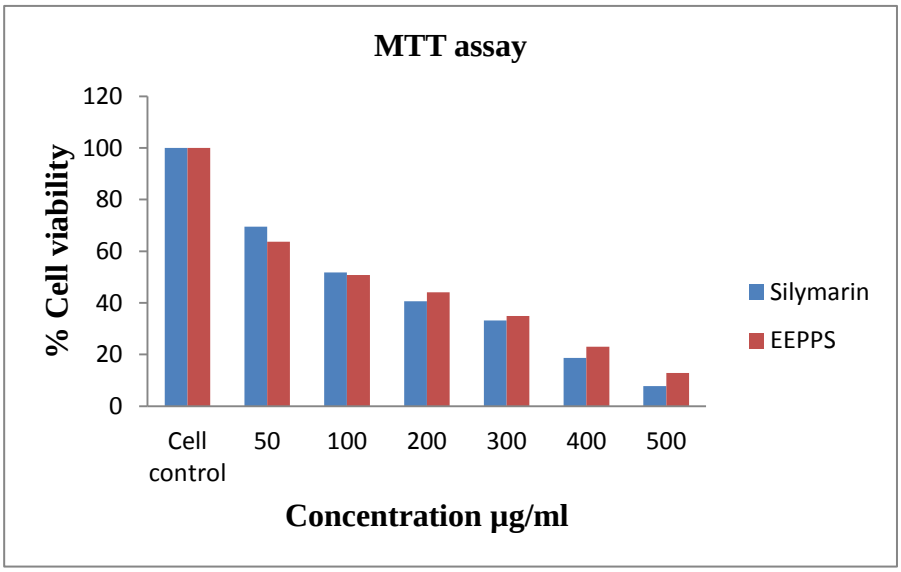
Table - 2

MTT assay of Silymarin and ethanolic extract of *Pongamia pinnata* L., seed on Chang liver cell line

S. No	CONCENTRATION (µg/ml)	%CELL VIABILITY	
		SILYMARIN	EEPPS
1.	50	69.49	63.71
2.	100	51.84	50.84
3.	200	40.67	44.06
4.	300	33.20	34.89
5.	400	18.64	23.03
6.	500	7.77	12.86
7.	Cell control	100	100

Figure. 5

Cytotoxicity of Standard Silymarin and extract on Chang liver cell line



The cell viability decreased with increase in concentration in the case of Silymarin and EEPPS. The CTC_{50} values of the EEPPS was found to be 100µg/ml which is similar to that of the standard Silymarin. This indicates that the EEPPS is as safe as Silymarin against the normal Chang liver cell line.

6.3.1.2. *In vitro* hepatoprotective activity using Standard Silymarin and extract against CCl_4 induced hepatotoxicity

The Chang liver cells were first challenged with the CCl_4 at the concentration of 125µg/ml in which 39.92% cells are viable. Then the cells were treated with standard Silymarin and ethanolic extract of *Pongamia pinnata* L., seed in the concentration of 100, 50, 25, 10µg/ml to assess the percentage cell viability and the values are tabulated in **Table 3 and Figure.6**

Table - 3

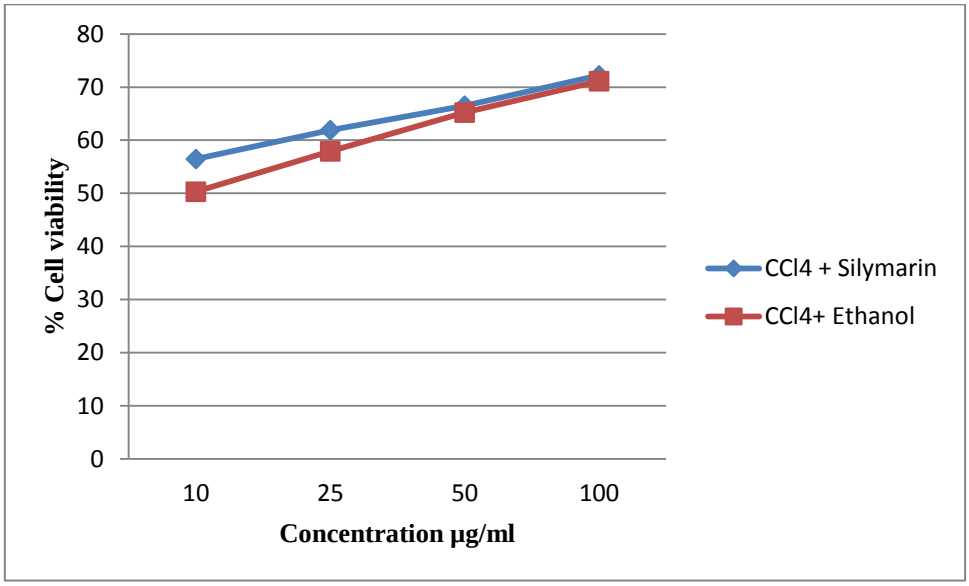
***In vitro* hepatoprotective activity of Silymarin and EEPPS against CCl_4 induced hepatotoxicity**

S. NO	TREATMENT	CONCENTRATION (µg/ml)	% CELL VIABILITY
1.	Control		100
2.	CCl_4	125	39.92±1.02
3.	CCl_4 + Silymarin	10	56.46±2.0
		25	61.9±2.3
		50	66.5±2.34
		100	72.2±2.1
4.	CCl_4 + EEPPS	10	50.3±0.54
		25	57.9±1.89
		50	65.2±2.32
		100	71.1±1.20

Values are expressed as Mean ± S.D (n=3)

Figure. 6

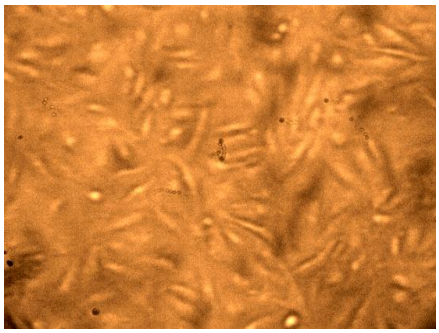
***In vitro* hepatoprotective activity of Silymarin and extract using CCl_4 induced toxicity**



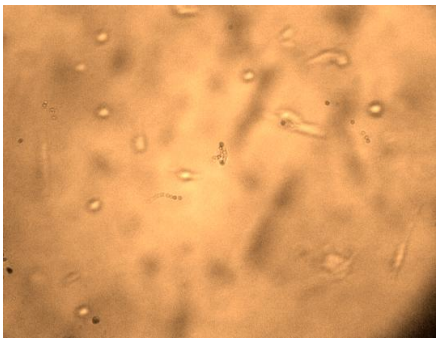
In the Silymarin treated group, the cell viability was 72.2% at the highest concentration of 100µg/ml. The EEPPS group showed in cell viability of 71.2% which is relatively close to that of the standard Silymarin. This indicates that the EEPPS has hepatoprotective activity comparable with that of Silymarin.

The Cell morphology of the Chang liver cells of different group are shown in Figure.7

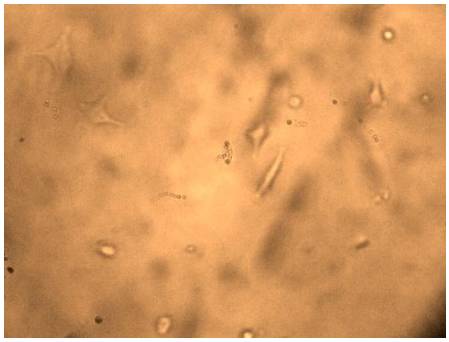
Figure.7



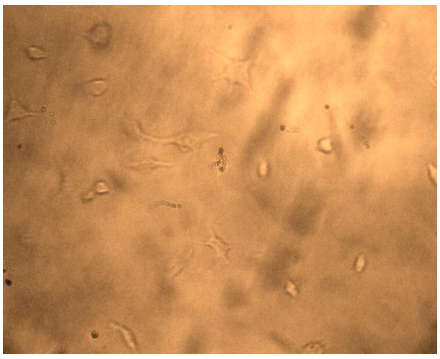
a) Normal Chang cell



b) Carbon tetrachloride



c) CCl_4 + Silymarin



d) CCl4 +Ethanol extract

The normal polygonal cell structure is seen in Figure a. In the CCl_4 treated group the polygonal cells have shrunk and appear round in shape indicating cytotoxicity. These changes are attenuated in the Silymarin and EEPPS groups.

6.4. *IN VIVO* STUDIES

6.4.1. *IN VIVO* HEPATOPROTECTIVE EVALUATION OF EEPPS USING CCl_4 MODEL OF HEPATOTOXICITY

Hepatoprotective activity

Body weight

The body weight of animals was determined on 1st and 15th day of the study period and these are tabulated in **Table 4 and Figure.8**

Table - 4

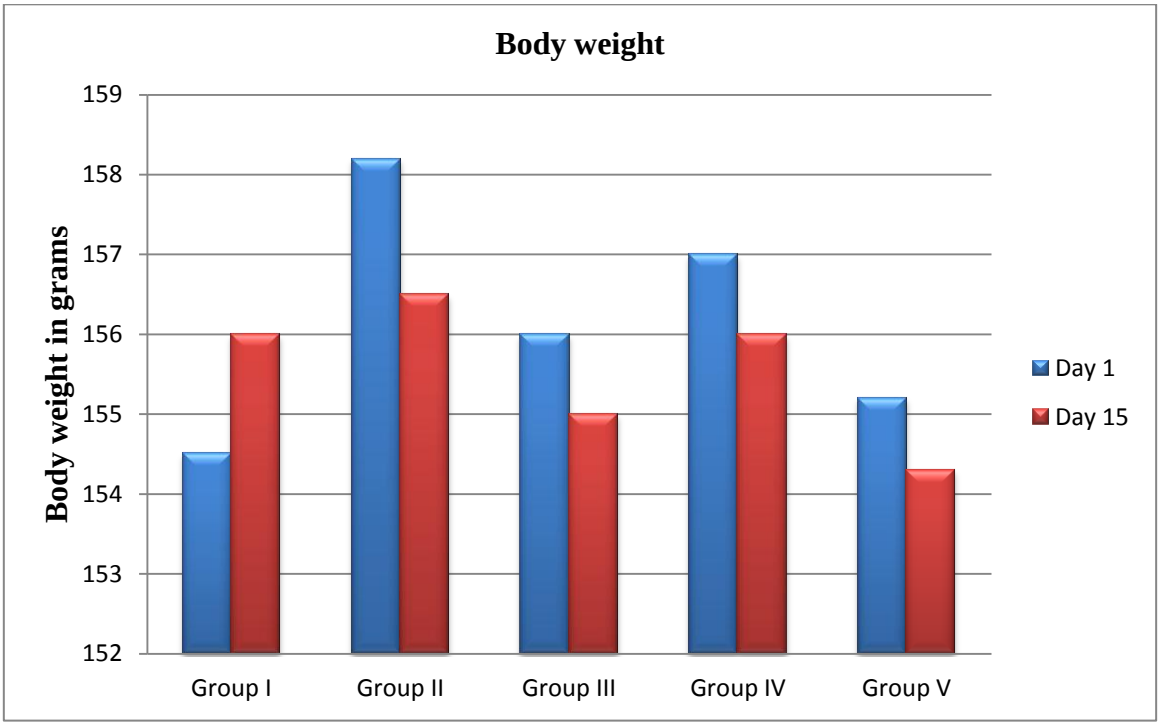
Body weight of animals in the various groups

GROUPS	TREATMENT	ANIMAL BODY WEIGHT IN gms	
		1st day	15th day
I	Control	154.5±2.92	156±2.28
II	Disease control	158.2±2.19	156.5±2.85
III	Silymarin (25mg/kg)	156±3.85	155±3.54
IV	*Pongamia pinnata* L., (200mg/kg)	157±2.29	156±2.49
V	*Pongamia pinnata* L., (400mg/kg)	155.2±3.21	154.3±2.92

Values are expressed as Mean ± S.D (n=6)

Figure. 8

Body weight of animals in the various groups



It is seen from the data that in the CCl_4 treated group there was a slight decrease in body weight on the 15th day as compared to 1st day. In the Silymarin and EEPPS treated group the reduction in body was lesser than that of CCl_4 group.

6.5. BIOCHEMICAL PARAMETERS

6.5.1. Aspartate Aminotransferase (AST) evaluation

The AST levels of the animals treated with CCl_4 alone and those that were given CCl_4 and Silymarin/ EEPPS were estimated on Day 15. They are tabulated in **Table 5 and Figure.9**

Table - 5

GROUP	TREATMENT	AST (U/ml)
I	Control	84.8±3.62
II	Disease control	158±6.69*
III	Silymarin (25mg/kg)	94.5±4.03**
IV	*Pongamia pinnata* L., (200mg/kg)	135±3.36**
V	*Pongamia pinnata* L., (400mg/kg)	103±6.77**

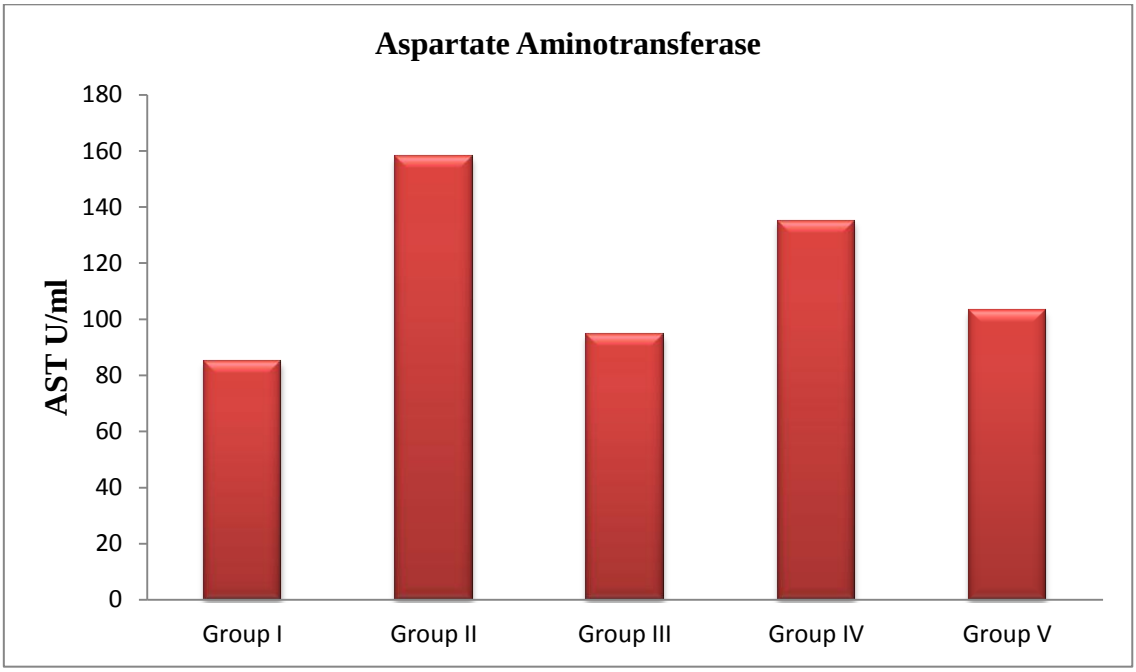
The values are expressed as mean ± S.D (n=6)

*P<0.001 compared to control group.

**P<0.001 compared to disease control group.

Figure. 9

AST levels



It was seen that the AST levels had increased significantly in animals which were given CCl_4 as compared to normal group. Treatment with Silymarin showed a significant decrease in the elevated levels of AST. The EEPPS treated groups of animals also showed a significant decrease in the elevated levels of AST. The reduction was more in the group treated with the higher dose (400mg/kg) of EEPPS.

6.5.2. Alanine Aminotransferase (ALT) evaluation

The ALT levels of the animals treated with CCl_4 alone and those that were given CCl_4 and Silymarin and EEPPS were estimated on Day 15.They are tabulated in **Table 6 and Figure.10**

Table - 6

GROUP	TREATMENT	ALT (U/ml)
I	Control	80.5±7.08
II	Disease control	182.6±14.4*
III	Silymarin (25mg/kg)	69.6±2.42**
IV	*Pongamia pinnata* L., (200mg/kg)	96.3±6.34**
V	*Pongamia pinnata* L., (400mg/kg)	83.3±2.92**

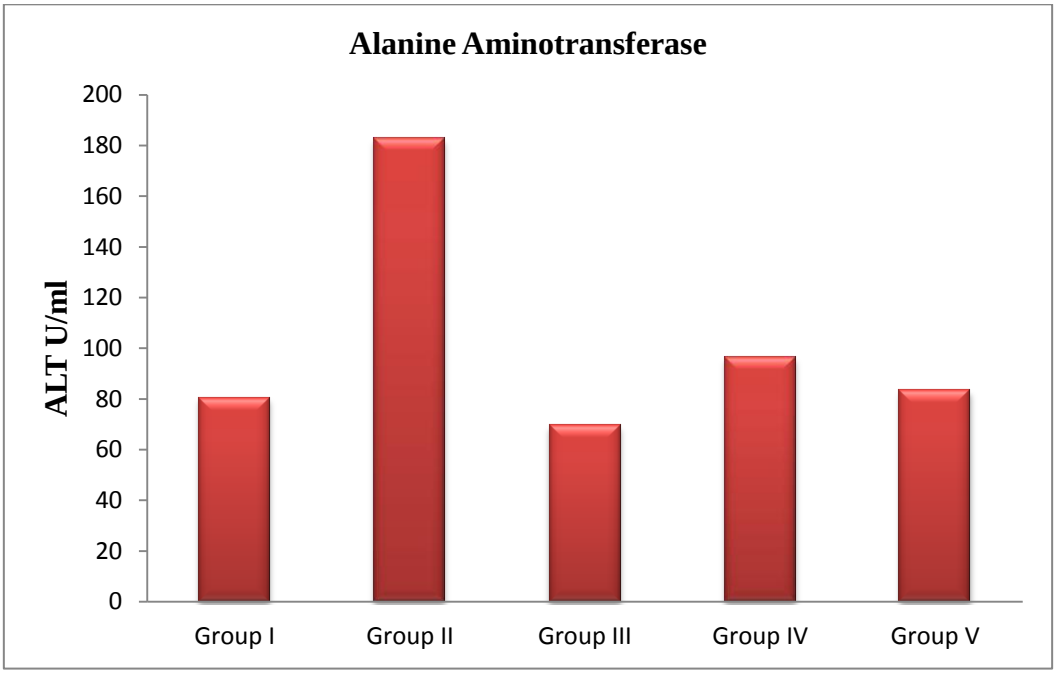
The values are expressed as mean ± S.D (n=6)

*P<0.001 compared to control group.

**P<0.001 compared to disease control group.

Figure.10

ALT levels



It was seen that the ALT levels had increased significantly in animals which were given CCl_4 as compared to normal group. Treatment with Silymarin showed a significant decrease in the elevated levels of ALT. The EEPPS treated groups of animals also showed a significant decrease in the elevated levels of ALT. The reduction was more in the group treated with the higher dose (400mg/kg) of EEPPS.

6.5.3. Alkaline Phosphatase (ALP) evaluation

The ALT levels of the animals treated with CCl_4 alone and those that were given CCl_4 and Silymarin/ EEPPS were estimated on Day 15. They are tabulated in **Table 11and Figure.7**

Table - 7

GROUP	TREATMENT	ALP (U/ml)
I	Control	138±9.42
II	Disease control	202±14.8*
III	Silymarin (25mg/kg)	110±10.4**
IV	*Pongamia pinnata* L., (200mg/kg)	183±9.29***
V	*Pongamia pinnata* L., (400mg/kg)	123.1±6.51**

The values are expressed as mean ± S.D (n=6)

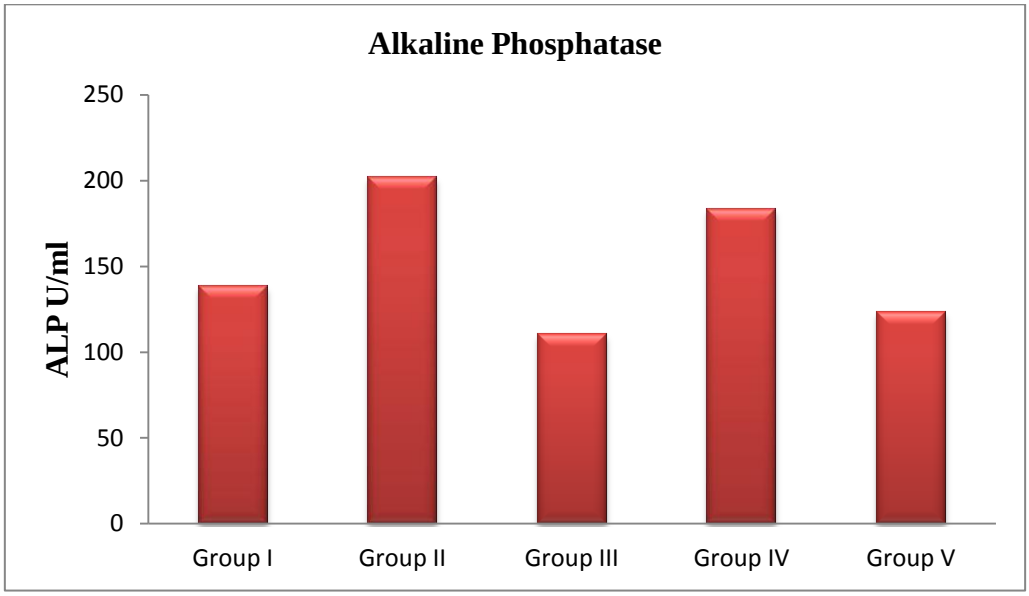
* P<0.001 compared to control group.

** P<0.001 compared to disease control.

. *** P<0.05 compared to disease control.

Figure.11

ALP levels



It was seen that the ALP levels had increased significantly in animals which were given CCl_4 as compared to normal group. Treatment with Silymarin showed a significant decrease in the elevated levels of ALP. The EEPPS treated groups of animals also showed a significant decrease in the elevated levels of ALP. The reduction was more in the group treated with the higher dose (400mg/kg) of EEPPS.

6.5.4. Total Bilirubin (TB) evaluation

The Bilirubin levels of the animals treated with CCl_4 alone and those that were given CCl_4 and Silymarin/ EEPPS were estimated on Day 15. They are tabulated in **Table 12 and Figure. 8**

Table - 8

GROUP	TREATMENT	TOTAL BILIRUBIN (gm/dl)
I	Control	0.41±0.66
II	Disease control	1.13±0.28*
III	Silymarin (25mg/kg)	0.83±0.07**
IV	*Pongamia pinnata* L., (200mg/kg)	0.88±0.10**
V	*Pongamia pinnata* L., (400mg/kg)	0.85±0.05**

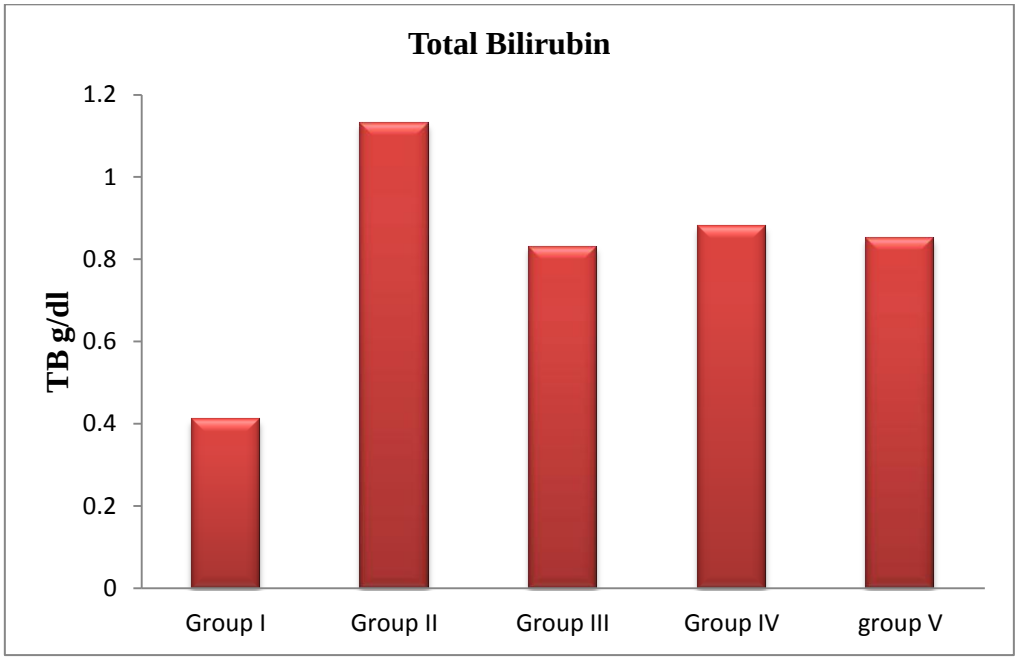
The values are expressed as mean ± S.D (n=6)

*P<0.001 compared to control group.

** P<0.05 compared to disease control.

Figure. 12

Bilirubin levels



It was seen that the Bilirubin levels had increased significantly in animals which were given CCl_4 as compared to normal group. Treatment with Silymarin showed a significant decrease in the elevated levels of Bilirubin. The EEPPS treated groups of animals also showed a significant decrease in the elevated levels of Bilirubin. The reduction was more in the group treated with the higher dose (400mg/kg) of EEPPS

6.5.5. Total Protein (TP) evaluation

The Total Protein levels of the animals treated with CCl_4 alone and those that were given CCl_4 and Silymarin/ EEPPS were estimated on Day 15. They are tabulated in **Table 13 and Figure. 9**

Table - 9

GROUP	TREATMENT	TOTAL PROTEIN (g/dl)
I	Control	7.58±0.24
II	Disease control	6.8±0.16*
III	Silymarin (25mg/kg)	6.95±0.28**
IV	*Pongamia pinnata* L., (200mg/kg)	6.45±0.29**
V	*Pongamia pinnata* L., (400mg/kg)	6.76±0.16**

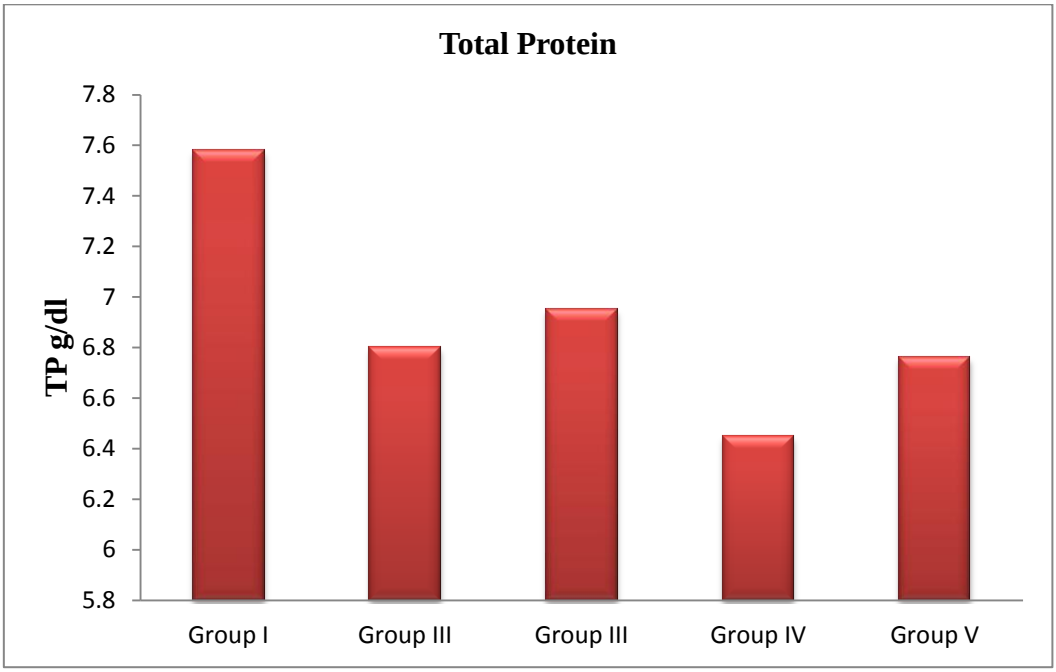
The values are expressed as mean ± S.D (n=6)

*P<0.001 compared to control group.

** P<0.05 compared disease group.

Figure. 13

Total Protein levels



It was seen that the Total Protein levels had decreased significantly in animals which were given CCl_4 as compared to normal group. Treatment with Silymarin showed a significant increase in the decreased levels of Total Protein. The EEPPS treated groups of animals also showed a significant increase in the decreased levels of Total Protein. The elevation was more in the group treated with the higher dose (400mg/kg) of EEPPS.

6.5.6. Albumin (ALB) evaluation

The Total Protein levels of the animals treated with CCl_4 alone and those that were given CCl_4 and Silymarin/ EEPPS were estimated on Day 15. They are tabulated in **Table 10 and Figure. 14**

Table - 10

GROUP	TREAMENT	ALBUMIN (Grams)
I	Control	3.8±0.25
II	Disease control	3.5±0.18*
III	Silymarin (25mg/kg)	3.7±0.12**
IV	*Pongamia pinnata* L., (200mg/kg)	3.6±0.1**
V	*Pongamia pinnata* L., (400mg/kg)	3.7±0.12**

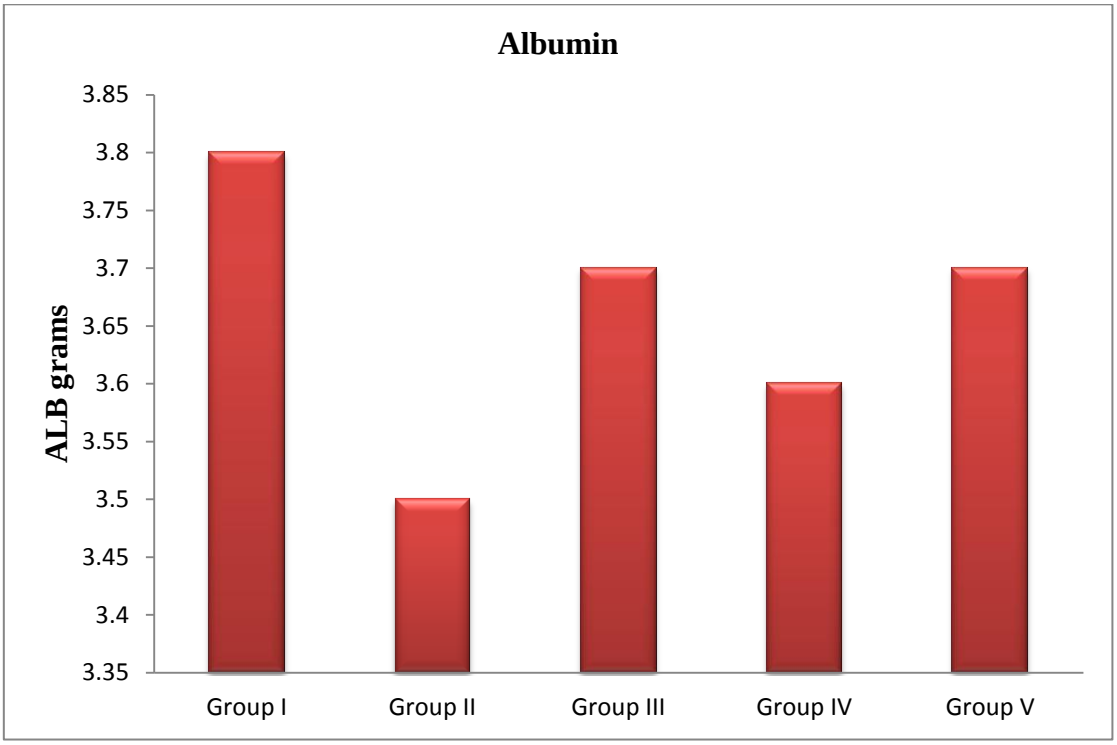
The values are expressed as mean ± S.D (n=6)

*P<0.001 compared to control group.

**P<0.05 compared to disease group.

Figure. 14

Albumin levels



It was seen that the Albumin levels had decreased significantly in animals which were given CCl_4 as compared to normal group. Treatment with Silymarin showed a significant increase in the decreased levels of Albumin. The EEPPS treated groups of animals also showed a significant increase in the decreased levels of Albumin. The elevation was more in the group treated with the higher dose (400mg/kg) of EEPPS.

6.6. ESTIMATION OF ANTIOXIDANT ENZYMES LEVEL

6.6.1.Lipid peroxidation (LPO)

The Lipid peroxidase enzyme levels of the animals treated with CCl_4 alone and those that were given CCl_4 and Silymarin/ EEPPS were esimated in liver homogenised solution on Day15. They are tabulated in **Table 11 and Figure. 15**

Table -11

GROUP	TREATMENT	LPO (moles/100mg/Protein)
I	Control	2.25±0.07
II	Disease control	6.50±0.04*
III	Silymarin (25mg/kg)	4.98±0.09**
IV	*Pongamia pinnata* L., (200mg/kg)	5.55±0.39**
V	*Pongamia pinnata* L., (400mg/kg)	4.64±0.03**

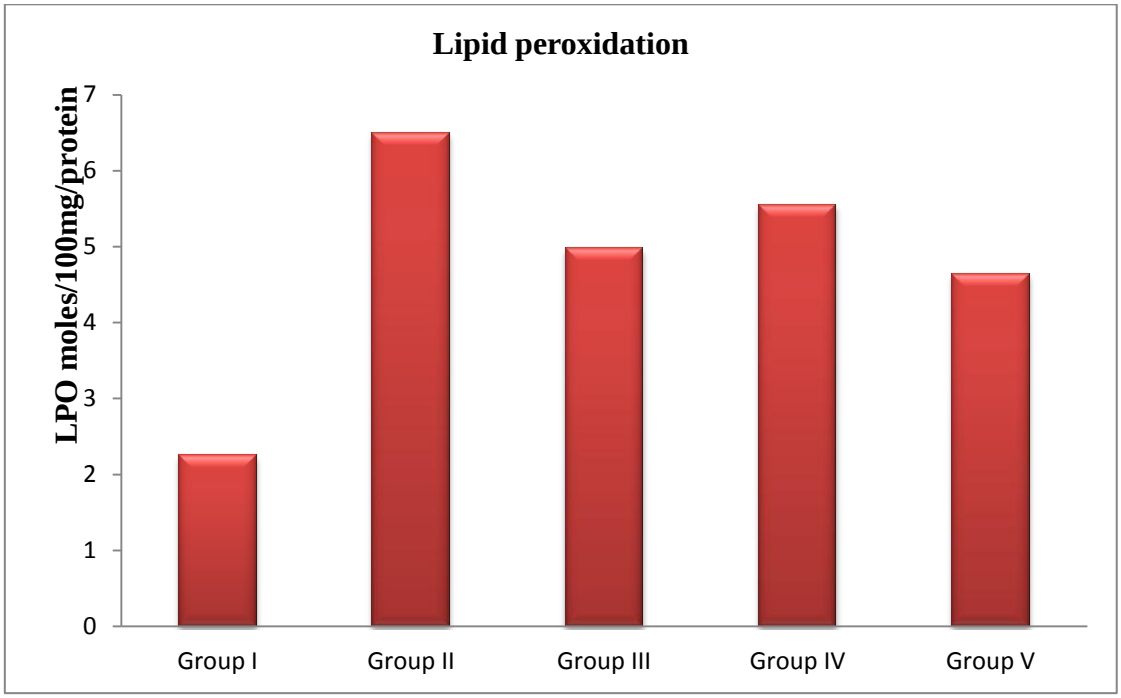
The values are expressed as mean ± S.D (n=6)

*P<0.001 compared to control group.

** P<0.001 compared disease group.

Figure. 15

LPO levels



It was seen that the LPO levels had increased significantly in animals which were given CCl_4 as compared to normal group. Treatment with Silymarin showed a significant decrease in the elevated levels of LPO. The EEPPS treated groups of animals also showed a significant decrease in the elevated levels of LPO. The elevation was more in the group treated with the higher dose (400mg/kg) of EEPPS.

6.6.2. Superoxide Dismutase (SOD)

The Superoxide dismutase enzyme levels of the animals treated with CCl_4 alone and those that were given CCl_4 and Silymarin/ EEPPS were esimated in liver homogenised solution on Day15. They are tabulated in **Table 12 and Figure.16**

Table – 12

GROUP	TREATMENT	SOD (Unit/min/mg of Protein)
I	Control	7.17±0.02
II	Disease control	5.91±0.03*
III	Silymarin (25mg/kg)	6.28±0.03**
IV	*Pongamia pinnata* L., (200mg/kg)	4.46±0.20**
V	*Pongamia pinnata* L., (400mg/kg)	5.58±0.03*

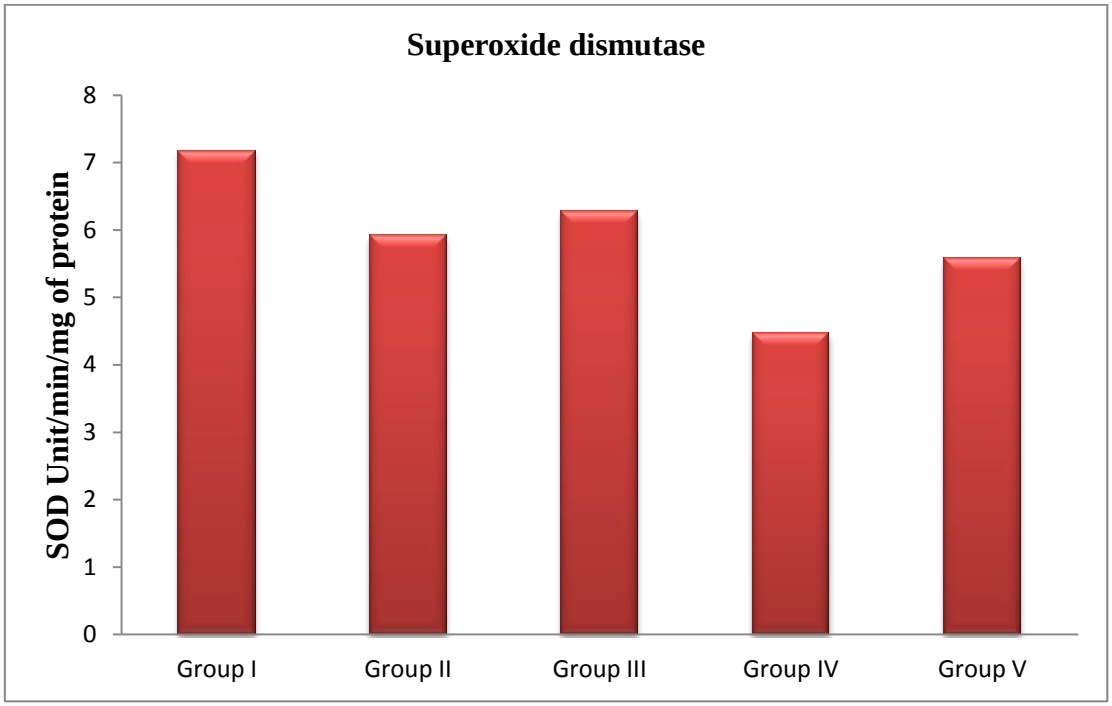
The values are expressed as mean ± S.D (n=6)

*P<0.001 compared to control group.

**P<0.001 compared disease group.

Figure. 16

SOD levels

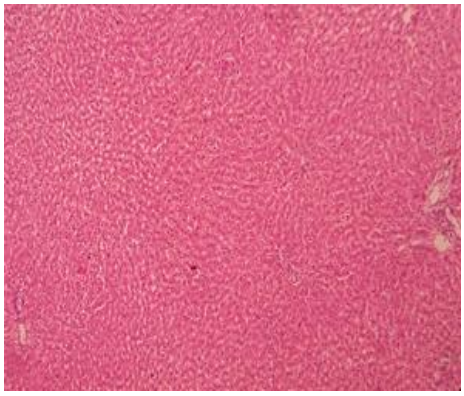


It was seen that the SOD levels had decreased significantly in animals which were given CCl_4 as compared to normal group. Treatment with Silymarin showed a significant increase in the decreased levels of SOD. The EEPPS treated groups of animals also showed a significant increase in the decreased levels of SOD. The elevation was more in the group treated with the higher dose (400mg/kg) of EEPPS.

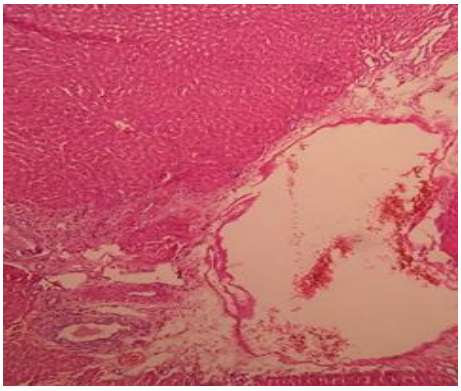
6.7. The histopathology of the liver was carried out the photograph of the slides are given in Figure.17

Figure. 17

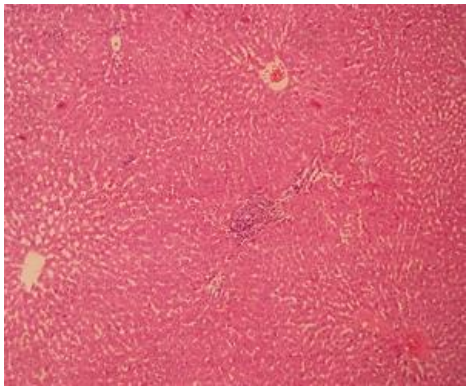
Histopathological studies of liver



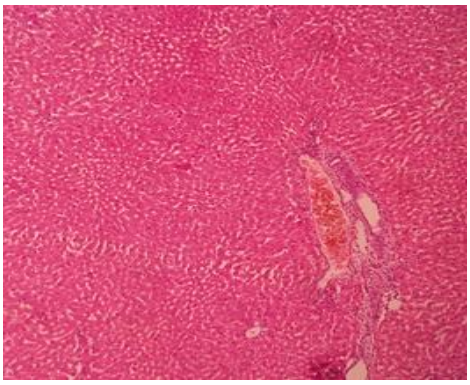
Normal control



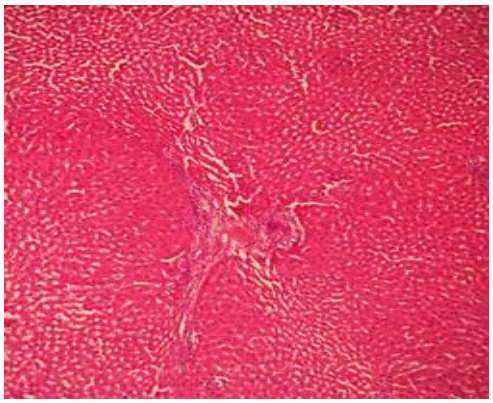
CCl_4 treated



CCl_4 + Silymarin 25mg/kg



CCl_4 + *Pongamia pinnata* L., 200mg/kg



CCl_4 + *Pongamia pinnata* L., 400mg/kg

8. DISCUSSION

Liver is an important organ involved in metabolism of many xenobiotics. It removes toxins from the body. It is also exposed to several drugs and xenobiotics which cause hepatic damage. In the present study hepatoprotective activity on the seeds of *Pongamia pinnata* L., was evaluated.

In vitro studies are carried out to evaluate the safety of the drug under study before taking them up for *in vivo* evaluation.

The *in vitro* toxicity study was conducted on the ethanolic extract of *Pongamia pinnata* L., seeds using normal Chang liver cell line. The Chang liver cell line is differentiated non-malignant liver epithelial cell of human origin. The studies indicate that the EEPPS is as safe as Silymarin both having the same CTC_{50} of approximately 100µg/ml which is a standard drug.

After establishing the safety of EEPPS, *in vitro* hepatoprotective evaluation was done. *In vitro* studies with cultured cell lines or primary hepatocytes have proved to be promising methods to evaluate hepatoprotective activity. For this study, CCl_4 mediated hepatotoxicity on Chang liver cell line was used. CCl_4 is the most commonly used hepatotoxin in the study of free radical induced liver disease.

For *in vitro* evaluation, the hepatoprotective activity of an agent can be established by MTT assay and also by observing the morphological changes in the cell.

For MTT assay different concentrations (10, 25, 50, 100µg/ml) of Silymarin and EEPPS were used. In the group challenged with CCl_4, the cell viability was only 39.9%.The cell viability of the CCl_4 and EEPPS treated group was 71.2 % the cell viability of the CCl_4 and Silymarin treated group which was 72.2%. These data established that the EEPPS has a good hepatoprotective activity which is comparable to Silymarin, the currently used hepatoprotective drug. So confirm the hepatoprotective activity of the EEPPS, *In vivo* studies was carried out using CCl_4 induced hepatic damage in rats.

CCl_4 - induced hepatic injury is an experimental model widely used for the screening of hepatoprotective drugs. CCl_4 undergoes a biotransformation by hepatic microsomal cytochrome P-450 to produce trichloromethyl free radicals. These hepatotoxic metabolite can react with protein and lipid in the membrane of cells or organelles leading to necrosis of hepatocytes. As a result of hepatic injury, permeability of the cell membrane is altered causing the cytosolic transaminase (ALT, AST) in the circulation. Hence evaluations of AST and ALT are definite indicators of hepatoprotective activity. The rise in the serum levels of ALP, AST, ALT and bilirubin as observed in the present study could be attributed to the damaged structural integrity of the liver. Liver damage is also associated with elevated levels of ALT, and Bilirubin. It is also associated with decrease in levels of Total Protein and Albumin.

The administration of EEPPS at both doses 200mg/kg and 400mg/kg showed a improvement in the biochemical parameter profile of the animals. The effect seen with the higher dose is almost equal to that of the standard drug Silymarin. These evaluation study confirms the hepatoprotective potential of EEPPS.

It has been hypothesized that one of the principal causes of CCl_4- induced liver injury is formation of lipid peroxidases by free radical derivatives of CCl_4 ($CCl_{3.}$). Thus, the antioxidant activity or the inhibition of the generation of free radicals is important in the protection against CCl_4-induced hepatotoxicity.

The body has an effective defence mechanism to prevent and neutralize the free radical-induced damage. This is accomplished by a set of endogenous antioxidant enzymes such as SOD, Catalase. SOD has been reported as one of the most important enzyme in the enzymatic antioxidant defense system. It scavenges the superoxide anion to form hydrogen peroxide and thus diminishing the toxic effect caused by this radical. Decrease in enzyme activity of superoxide dismutase (SOD) is a sensitive index in hepatocellular damage and is the most sensitive enzymatic index in live injury. The EEPPS causes a significant increase in hepatic SOD activity indicating a reduction of reactive free radical induced oxidative damage to liver.

Lipid peroxidation has been implicated in the pathogenesis of hepatic injury by compounds like CCl_4 and is responsible for cell membrane alteration. In the present study, elevated level of LPO observed in CCl_4 administered rats indicated excessive formation of free radicals and activation of LPO system resulting in hepatic damage. Treatment with different dose levels of EEPPS significantly reversed these changes. Hence, it is possible that the mechanism of hepatoprotection of *Pongamia pinnata* L., might be due to its antioxidant action.

Histopathological liver sections also revealed that the hepatic architecture was altered by hepatotoxin in Carbon tetrachloride group, whereas in the liver sections of the rat treated with the EEPPS and intoxicated with CCl_4, the hepatic architecture was not altered and was comparable with the standard drug Silymarin. The histopathological study confirms the significant hepatoprotective effect of ethanolic extract of *Pongamia pinnata* L., seeds especially at the dose 400mg/kg.

9. CONCLUSION

From the study it is concluded that

- The ethanolic extract of the seeds of *Pongamia pinnata* L., showed *in vitro* hepatoprotective activity against CCl_4 induced toxicity in Chang liver cell line.
- The ethanolic extract of *Pongamia pinnata* L., seeds possesses promising hepatoprotective activity against CCl_4 induced hepatic damage. The activity is more pronounced at a dose of 400mg/kg.
- The activity could be due to improvement in the antioxidant enzyme levels and a decrease in free radical levels. The presences of phytochemicals such as flavonoids have been shown to be responsible for hepatoprotective activity.
- Further studies can be carried out in the future to elucidate the mechanism of action of the ethanolic extract of seeds of *Pongamia pinnata* L., which may then be followed by and then clinical studies to establish its efficacy in humans.

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