CERTIFICATE

This is to certify that the Dissertation work entitled "**PHYTOCHEMICAL AND PHARMACOLOGICAL EVALUATION OF ETHANOLIC EXTRACT OF *LAWSONIA INERMIS***" submitted to The Tamilanadu Dr. M.G.R Medical University, Chennai, is a bonafide project work of University **Reg No: 26119231** carried out in the Department of Pharmacology, Cherraan's College of Pharmacy, Coimbatore for the partial fulfillment for the degree of **Master of Pharmacy** in **Pharmacology** under my guidance during the academic year 2013-2014.

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Mr. Gurumani M.Pharm.,(Ph.D),
Asst. Professor,
Department of Pharmacology,
Cherraan's College of Pharmacy.

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Dr. N. Thirumoorthy, M.Pharm,Ph,D,
Principal,
Cherraan's College of Pharmacy.

DECLARATION

The research work embodied in this work "**PHYTOCHEMICAL AND PHARMACOLOGICAL EVALUATION OF ETHANOLIC EXTRACT OF *LAWSONIA INERMIS***" was carried out in Department of Pharmacology, Cherraan's College of Pharmacy, Coimbatore under the direct supervision of **Mr.M.Gurumani M.Pharm, (Ph.D),** Asst.Professor, Department of Pharmacology, Cherraan's College of Pharmacy, Coimbatore-39.

This Dissertation submitted to the Tamilnadu Dr.M.G.R.Medical University, Chennai, for the award of degree of **Master of Pharmacy** in **Pharmacology** during the academic year of 2013-2014.

Place: Coimbatore.

Date: University Reg. No **26119231**

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I submit my sincere thanks to our Chairman **Mr.K .C. PALANISAMY** Chairman Cherraan's foundation trust, for providing all the facilities to carry out this thesis work.

With the immense pleasure and pride, I would to take opportunity in expressing my deep sense of gratitude to our beloved guide, **Mr.M.Gurumani M.Pharm.,(Ph.D),**. Asst.Professor, Department of Pharmacology Cherraan's college of Pharmacy under whose active guidance, innovate ideas, constant inspiration and encouragement of the work entitle **"PHYTOCHEMICAL AND PHARMACOLOGICAL EVALUATION OF ETHANOLIC EXTRACT OF *LAWSONIA INERMIS*"** has been carried out.

My sincere gratitude to our beloved Principal **Dr. N. Thirumoorthy, M.Pharm,Ph,D,** Cherraan's College of Pharmacy for his encouragement and also providing all facilities in this institute to the fullest possible extent enabling us to complete this work.

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I gratefully acknowledge the endless love and support of my **Father and Mother**. They are **my living Gods**, as who guided me in the rightful way to achieve all my activities. They gave me the incredible effort to become a successful person for bright future in this world. Thanks a lot to my **Parents**.

I am giving whole heart thanks to all my **Friends** and **Classmates** for their help and sharing during my project.

EVALUATION CERTIFICATE

This is certify that the Dissertation work entitled "**PHYTOCHEMICAL AND PHARMACOLOGICAL EVALUATION OF ETHANOLIC EXTRACT OF *LAWSONIA INERMIS***" submitted by University **Reg.No: 26119231** to The Tamilnadu Dr. M.G.R Medical University, Chennai, in the partial fulfillment for the degree of **Master of Pharmacy** in **Pharmacology,** is a record of bonafide work carried out by the candidate at the Department of Pharmacology, Cherraan's College of Pharmacy, Coimbatore and was evaluated by us during the academic year 2013-2014.

Internal Examiner **External Examiner**

Dedicated to

My

Beloved Parents

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LIST OF ABBREVIATION USED

b.w	-	Body weight.
CPCSEA	-	Committee for the Purpose of Control and Supervision of Experimental Animals.
Ext	-	Extract.
Fig	-	Figure.
g m	-	Gram.
IAEC	-	Institutional Animal Ethical Committee.
P.O	-	Per oral.
LD_{50}	-	Lethal dose.
Mtr	-	Meter.
No	-	Number.
ANOVA	-	Analysis of variance.
C	-	Degree of centigrade.
SEM	-	Standard Error Mean
Con	-	Conventration.
EELI	-	Ethanolic Extract of *Lawsonia Inermis Linn*
SGOT	-	Serum Glutamate Oxaloacetate Transaminase.
SGPT	-	Serum Glutamate Pyruvate Transaminase.
AST	-	Aspartate Amino Transferase.
ALT	-	Aspartate Alanine Transferase.
ALP	-	Alkaline Phosphate.
TB	-	Total Bilirubin.
TP	-	Total Protein.

INTRODUCTION

The greek word for liver is *hepar,* so medicinal terms related to liver often start with hapato or hepatic. liver is the one of the largest organ in the body and the cheif site for intense metabolism and excretion. It has a surprising role in the maintenance, performance and regulating homeostasis of the body. It is involved with almost all biochemical pathway to growth, fight against disease, nutrient supply, energy provision and reproduction. The major function of the liver are carbohydrate , protein and fat metabolism, detoxification, secretion of bile and storage of vitamin. Thus, to maintain a healthy liver is a crucial factor for overall health and well being. But when it is continuously and variedly exposed to environment toxins, chemicals like ccl_4, drug habits, alcohol, infection and auto immune disorders, prescribed (antibiotics, chemotherapeutic agents like paracetamol) cum over the counter drugs can eventually lead to various liver ailments like hepatitis, cirrhosis, and alcoholic liver disease[1].

Liver damage is always associated with cellular necrosis, increase in tissue lipid peroxidation and depletion in the tissue glutathione (GSH) levels. In addition, serum levels of many biochemical markers like serum glutamate oxaloacetate transaminase (SGOT/AST) and serum glutamate pyruvate transaminase (SGPT/ALT) triglycerides, cholesterol, bilirubin and alkaline phosphates are elevated.

The following are some of the liver diseases that are commonly observed,

a) Necrosis
b) Cirrhosis
c) Hepatitis – may be of viral, toxic or deficiency type.
d) Hepatic failure – acute or chronic
e) Liver disorder due to impairment of metabolic function. Generally the disorder associated with fat (liposis) and bilirubin (jaundice) metabolism are very commonly seen.

1. Disorder associated with fat metabolism: fatty liver
2. Disorder associated with bilirubin metabolism: jaundice or which may be of different types based upon mechanism of action and etiology.

a) Haemolytic/pre-hepatic jaundice.
b) Obstructive (post-hepatic/cholestatic jaundice).
c) Hepatogenous/hepatic jaundice/cholstasis.

In these three condition there occurs unconjucated hyperbilirubinaemia.

d) Hereditary jaundice or pure cholstasis: gilberts syndrome, dubin jhonson syndrome and crigler-naijar syndrome etc, rotors syndrome are some of the hereditary jaundice types.
e) Chemical/drug induced hepatotoxicity: generally may be hepatitis, jaundice and carcinogenesis.

Thus liver diseases are one of the fatal diseases in the world today. They pose a serious challenge to international public health. Modern medicines have little to offer for alleviation of hepatic diseases and it is chiefly the plant based preparation which are employed for the treatment of liver disorder. But there are not much drugs available for the treatment of liver disorders[2,3].

Herbal drugs provide significant source of hepatoprotective drugs. Mono and poly-herbal preparation have been used in various liver disorders. According to an estimate, more than 700 mono and poly-herbal preparations in the form of decoction, tincture, tablets and capsules from more than 100 plants are in clinical use as hepatoprotective[4].

Therefore , many folk remedies from plant origin are tested for its potential antioxidant and hepatoprotective liver damage in experimental animal model. Several authors have reported favourable results with herbal drugs (mostly in the form of extracts) either in animals or in human studies. *Ginkgo biloba L., Echinacea purpurea L., hypericum perforatum L. and cimcifuga*

racemosa L Nutt. Were successfully subjected to clinical trials after preclinical evaluation.

Treatment option for common liver diseases such as cirrhosis, fatty liver, and chronic hepatitis are problematic. The effectiveness of treatment such as interferon, colchicines, pencillamine and corticosteroids are inconsistent at best and theincidence of side –effects profound. Too often the treatment is worse than the disease. Physicians and patients are in need of effective therapeutic agent with a low incidence of side effects. Plants potentially constitute such agroup. Several hundred plants have been examined for use in a variety of liver disorders[5,6].

The latter category of plants include:

- *Silybum marianum* (Milk thistle)
- *Picorrhiza kurroa* (Kutkin)
- *Curcuma longa* (Turmeric)
- *Camellia sinensis* (Tea)
- *Glycyrrhiza glabra* (Liqourice)
- *Allium sativum* (Garlic)
- *Phyllanthus niruri and Eclipta alba*

These plants are hepatoprotective medicinal herbs, which have shown genuine utility in liver disordes. These plants are used widely in hepatoprotective preparations and extensive studies have been done on them. There are number of phytoconstituents from plants which have exhibited hepatoprotective activity. Recent progress in the study of such plants has resulted in the isolation of about 170 different phytoconstituents from plants belonging to about 55 families, which exhibit hepatoprotective activity.

HEPATOTOXICITY

Hepatotoxin is a toxic substance which damages the liver. Toxic liver injury produced by drugs and chemicals may virtually mimic any form of naturally occurring liver disease. Hepatoprotective effect was studied against chemicals and drugs induced hepatotoxicity in rats like alcohol, carbon tetra chloride, galactosamine, paracetamol, isoniazide and rifampicin, antibiotics, peroxidised oil, aflatoxin etc.

Severiety of hepatotoxicity is greatly increased if the drug is continued after symptoms develop. Among the various inorganic compounds producing hepatotoxicity are arsenic, phosphorus, copper and iron. The organic agents include certain naturally occurring plant toxins such as pyrrozolidine, alkaloids, myotoxins and bacterial toxins[7].

Liver injury caused by hepatotoxins, such as carbon tetra chloride (CCL4), ethanol, and acetaminophen is characterised by varying degree of hepatocyte degeneration and cell death via either apoptosis or necrosis. The generation of reactive metabolites from the metabolism of hepatotoxins and the occurrence of reactive oxygen species (ROS) during the inflammatory reaction, account for the variety of pathophysiolocal pathways leading to cell death, such as covalent binding, disordered cytosolic calcium homeostasis, GSH depletion, onset of mitochondrial permeability transition (MPT) and associated lipid peroxidation. The metabolism of hepatotoxins by cytochrome P-450 enzyme subtypes is a key step of intoxication; therefore, enzyme inhibitors are shown to minimize the hepatotoxin-associated liver damage. Moreover, substantial evidence exists that MPT is involved in ROS-associated hepatocellular injury and few findings offer a novel therapeautic approach to attenuate cell damage by blocking the onset of MPT. Thus, oxidant stress and

lipid peroxidation are crucial elements leading to hepatotoxin-associated liver injury. In addition to specific treatment for given hepatotoxin, the general strategy for prevention and treatment of the damage includes reducing the production of reactive metabolites of hepatotoxins, using anti-oxidative agents and selectively targeting therapeutics to kupffer cells or hepatocytes for on –going processes, which play a role in mediating a second phase of injury[8].

CLASSIFICATION OF HEPATOTOXINE[9]

A. Intrinsic

It consist of agents that are predictable hepatotoxins. They are recognised by higeh incidence of hepatic injury exposed individuals and in experimental animals. There is a consistent latent period between exposure to a particular agent and the development of hepatic injury and the injury appeared to be dose related. There are two types of intrinsic hepatotoxins.

1. **Direct hepatotoxins**

It may be also called because they (metabolic products) produce direct ijury to hepatocytes and its organelles, especially the endoplasmic reticulam. Example carbon tetra chloride the proto type, produces peroxidation of the membrane lipids and other chemicals that lead to degeneration of the membranes.

2. **Indirect hepatotoxins**

They are anti-metabolites and related compounds that produce hepatic injury by interference with the specific metabolic pathway or processes. The structural injury produced by indirect hepatotoxins, appear to be secondary to a metabolic region. While in that produced by direct hepatotoxins, the metabolic dearrangement is secondary to the structural injury. The hepatic damage produce by indirect hepatotoxins may be mainly cytotoxic injury (by interfering with metabolic pathway or processes essential for parenchyma integrity) expressed as steatosis or necrosis, or may be mainly cholstasis, interfering only or mainly with biliary secretion.

B. Host idiosyncrasy

It consist of agents that are not predictably hepatotoxic, but produces hepatic injury in only a small portion of exposed individual. Who are uniquely susceptible. In several instance auto antibodies directed against normal cellular constituents are detected. The injury does not appear to be related and is not reproducible in experimental animals and appear after a variable latent period.

EVALUATION OF HEPATOPROTECTIVE ACTIVITY[10]

A review of literature reveals that several chemical substances and drugs having specific action on liver are used as hepatotoxins in experimental animals to stimulate ideal diseased conditions. The hepatoprotective activity can be most easily evaluated / screened with the aid of several model systems of liver damage in experimental animals.

In all test model system, condition for liver damage are implemented and an attempt is made to counteract this toxicosis with the substance preparation under test. The magnitude of the protective effect can be measured by estimating the enzyme activities and the rate of survival and can be verified with histologically. The available methods are *in-vivo, ex-vivo and in-vitro* methods. All these methods are used to study the protective or curative effect of any compound under test. In order to test for hepatoprotective activity the test substance and the hepatotoxin are administered simultaneously whereas in case of anti hepatoxicity or curative activity the test substance is generally administered after induction of hepatotoxicity.

A. In vitro methods

Hepatocytes are generally isolated by using *in-situ,* two steps recirculating collagenase perfution technique.these are then seeded in small containers and exposed to test samples and toxins. After a specified time period, the degree of toxicity or protection is assessed by viability tests and enzyme levels such as GOT and GPT. By employing primary culture hepatocytes using ccl4, galatosamine, thioacetamide, ethanol, paracetamol (PCML) etc, as hepatotoxins several hepatoprotective screening models have been devised. These have a number of advantages over *in-vivo* methods such as their ability to dispose numerous sample at a time, low cost with a small size, little variation and reproducibility of results. The major disadvantage is that sometimes it may not reflect the events which occur in animals.

B. In vivo methods

This method is used only to study the nature of the given compound but also to study the mechanism of the toxicant. Hepatotoxicity is produced in experimental animals by the administration of known dose of hepatotoxins like CCL4, galactosamine, thioacetamide, ethanol and paracetamol etc., which produced marked measurable effects, the magnitude of which can be measured by carrying out of various liver function tests viz. Morphological, metabolic or functional, biochemical and histopathological determinations. Although it is a very convenient laboratory method, reproducibility of results is rather poor. The compounds having hepatoprotective claims are also evaluated in general for their choleretic or anti-cholestatic activity in order to know whether the liver disorder is due to an abnormality of bilurubin metabolism or not. Choleretics are those agents which increase the out puts of bile by stimulating the liver where as anti-cholestatis are those which correct the retension and accumulation of bile duo to intrinsic and

extrinsic factors in the liver. These activities are evaluated by studying bile flow content in conscious and anaesthetized by animals for 5 hours

C. Ex vivo methods

In this models, after completion of preselected *in-vivo* test protocol hepatocytes are isolated and the percentage of viable cells and biochemical parameters are determined as liver function tests. These methods are somewhat better correlated to clinical models than *in-vitro* or *in-vivo* methods.

EXPERIMENTAL MODEL FOR HEPATOPROTECTIVE SCREENING

Several chemical reagents and drugs which induce liposis, necrosis, cirrhosis, carcinogenesis and hepatobiliary dysfunction in experimental animals are classified as hepatotoxins. The following are the some of the experimental models explained by employing some of the important hepatotoxins.

1. Paracetamol induced hepatotoxicity[11,12].
2. Carbon tetra chloride induced hepatotoxicity[13,14,15,16]
3. D-galactosamine induced hepatotoxicity[17].
4. Chloroform induced hepatotoxicity[18].
5. Ethanol induced hepatotoxicity[19,20,21].
6. Thioacetamide induced hepatotoxicity[22].

PARACETAMOL INDUCED HEPATOTOXICITY[11,12]

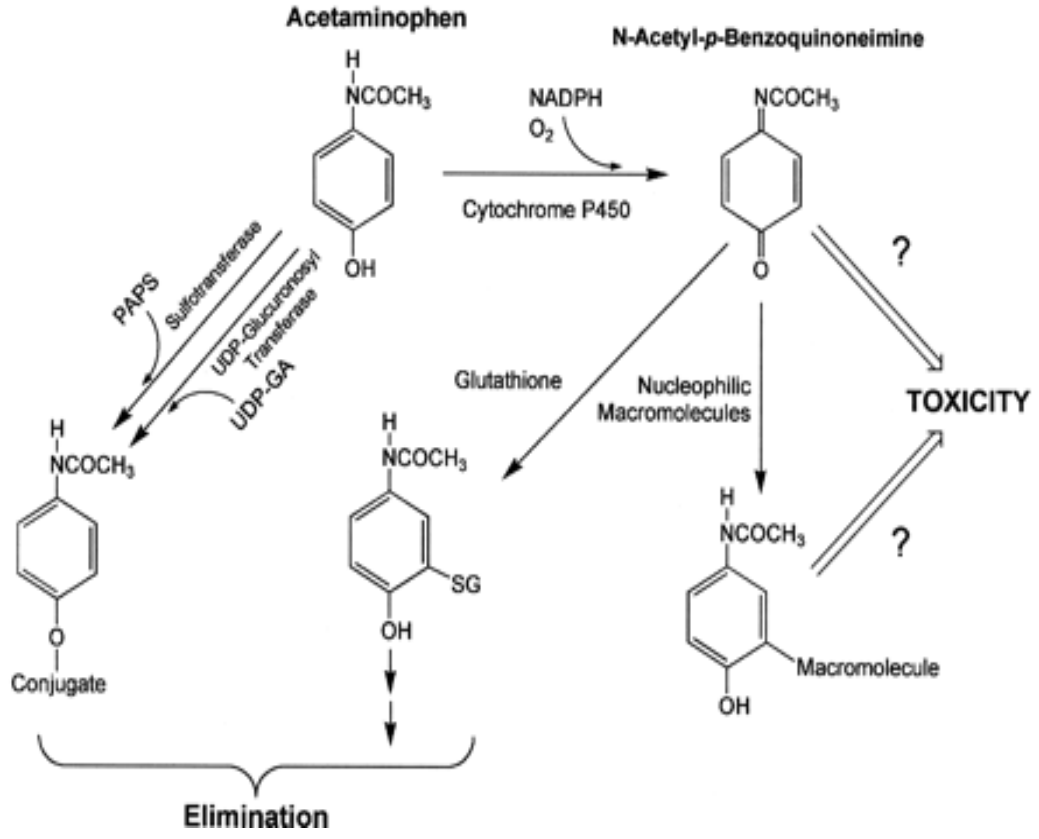
Paracetamol or acetaminophen (N-acetyl-p-aminophenol, APAP) induced hepatic injury are commonly used models for screening of hepatoprotective drugs and it causes acute centriobular necrosis in rats, mice, guinea pig, hamsters, rabbits, cats, dogs and pigs and centrizonal heamorrhagic necrosis in human mostly characterised by pyknosis and eosinophillic cytoplasm.

Paracetamol is one of the most widely used drug for analgesic and antipyretic activity worldwide. It one of the common pharmaceutical associated with both intentional accidental poisoning. It is a major cause of liver failure and causes death when taken in excess and is assumed to be safe inn recommended doses. It produces hepatic necrosis at higher doses. Paracetamol is rapidly absorbed from the stomach

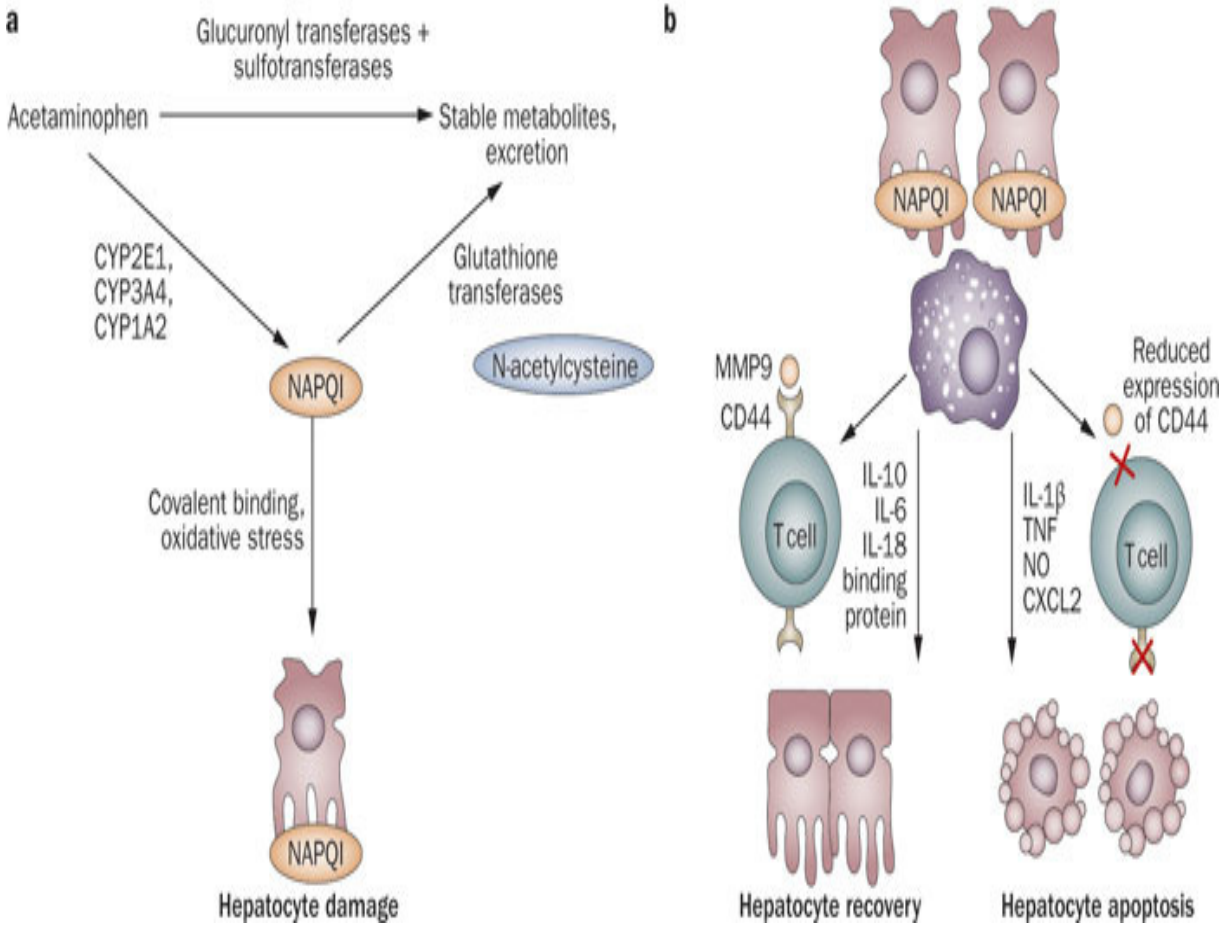
and small intestine and metabolised by conjugation in the liver to non-toxic agents. Therapeutic doses of drug are metabolised to sulphate and glucronide conjugates. The rest is metabolised to a reactive intermediate which is detoxified by conjugation with glutathione. In acute overdose or when the maximum daily dose is exceeded over a prolonged period, the normal conjugative pathway of metabolism becomes saturated. Excess paracetamol is then oxidatively metabolised in the liver via the mixed function oxidase P-450 system to a toxic metabolite N-acetyl-P-benzo-quinoneimine (NAPQI). NAPQI has extremely, short half life and is rapidly conjugated with glutathione, a sulfhydryl donor. Under condition of excessive NAPQI formation or reduced glutathione store, NAPQI covalently binds to vital proteins and the lipid bilayer of heatocyte membranes. The result is hepatocellulor death and cenrilobular liver necrosis. Small are eliminated by conjugation followed by excretion but when the conjugation enzymes are saturated, the drug is diverted to an alternative metabolic pathway, resulting in the formation of a hydroxylamine derivative by cytochrome P-450 enzyme. The hydroxylamine derivative, a reactive electrophillic agent, reacts non enzymatically with glutathione reacts with macromolecules and disrupts their structure and function. Extensive liver damage by paracetamol itself decreases its rate of metabolism and other substrates for hepatic microsomal enzymes. Induction of P-450 or depletion of hepatic glutathione is a prerequisite for paracetamol induced toxicity. In over dose, the sulphate and glucuronide conjugation pathways are saturated and more drugs are converted to the reactive metabolites accumulated bind covalently to liver cell proteins, causing irreversible damage. Liver damage can be prevented by providing glutathione like substances, such a acetylcystine, so that the reactive metabolite can be removed by conjugation and the liver cells are protected. An alternative view is that oxidative stress has a role in hepatotoxicity. There are many characteristic features of oxidative

stress in APAP hepatotoxicity, including lipidperoxidation, mitochondrial damage, ATP depletion, and formation of nitrotyrosine adducts in proteins, presumably owing to formation of superoxide-derived peroxynitrite. However, these processes may be consequences of the damage mediated by protein adduction rather than the direct effect of hepatotoxicity.

PARACETAMOL-INDUCED HEPATOTOXICITY



PATHOPHYSIOLOGY OF HEPATOXICITY:



ANALYSIS OF BIO CHEMICAL PARAMETER TO ASSESS LIVER FUNCTION

In keeping with the multiplicity of the liver, a variety of tests are available to assess them. The choice of the test is influenced by its simplicity, reliability, and sensitivity as well as the particular function, one is interested in assessing.

1. Serum enzyme parameters

Transaminases

A transamination is a process in which an amino group is transferred from an amino acid to alpha- keto acid. It is an important step in the metabolism of amino acids. The enzyme responsible for transamination are called transaminases or samino-transferases. Two diagnostically useful transaminases are glutamate oxaloacetate transaminase SGOT/SAT and glutamate pyruvate trasaminase SGPT/ALT. These two enzymes are sensitive markers of hepato-cellular injury.

A. Estimation of SGOT/AST[22,23,24]

Principle

SGOT (AST) catalyses the transfer of amino group SGOT is an enzyme found mainly in heart muscle, liver cells, skeletal muscle and kidneys. Injury to these tissues results in the release of the enzyme in blood. Elevated levels are found in myocardial infarction, cardiac operations, hepatitis, cirrhosis, acute pancreatitis, acute renal diseases and primary muscle diseases. Decreased levels may be found in pregnancy, Beri Beri and Diabetic ketoacidosis. Its normal serum level is 5-40 units/I.

between L-Aspartate and a ketoglutarate to form oxaloacetate and glutamate. The oxaloectate formed reacts with NADH in the presence of malate dehydrogenase to form NAD. The rate of oxidation of NADH to NAD is measured as a decrease in absorbance which is proportional to the SGOT (AST) activity in the sample.

AST

L-Aspartate + α-ketoglutarate ----------> oxaloacetate + L-Glutamate

MDH

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

SGOT levels are 10-200 fold elevated in patients with acute hepatic necrosis, viral hepatitis and drug induced poisoning. SGOT levels are also elevated by 10 fold in patient of post hepatic jaundice, intra hepatic cholestasis and less than 10 fold in alcoholic and hepatic steatosis. Very high levels are seen in extensive acute hepatic necrosis such as in severe viral hepatitis and acute cholestasis.

B. Estimation of SGPT/ALT[25,26,27]

SGPT or ALT is a cytosolic enzymes primarily present in the liver. Its normal serum level is 7-56 units/I.

Principle

SGPT (ALT) catalyses the transfer of amino group from L-alanine to alpha-ketoglutarate to yield pyruvate and L-glutamate. Lactate dehydrogenese then converts pyruvate and NADH into lactate and NAD. The conversion of NADH to NAD decreases the absorbtion at 340 nm. The rate of decrease in absorbance is measured and is proportional to the SGPT activity.

ALT

L-alanine + α-ketoglutarate **----------**> pyruvate + L-Glutamate

LDH

Pyruvate + NADH + H^+ ----------> L- Lactate + NAD^+

ALT activity is predominantly associated with liver tissues followed by comparatively lower levels in heart, muscle and kidneys. Quantitation of ALT is a useful parameter in evaluating liver function. Elevated levels of the enzymes are found in liver and kidney diseases such as infectious or toxic hepatitis, infectious mononucleosis and cirrhosis, moderate increase is also found in obstructive jaundice, metastasis carcinoma, hepatic congestion and myocardial infarction.

Phosphatases

Phosphatases belongs to the class of enzymes called hydrolases and they are characterized by their ability to hydrolyse a large variety of organic phosphates with the formation of an alcohol and phosphate ions.

Phosphatases of diagnostic significance are alkaline phosphatases and acid phosphatises. These are differentiated by their reaction in alkaline and acidic medium. The PH for measuring the alkaline phosphatase activity is 10 and for acid phosphatase is 5.

Alkaline phosphatase[28,29,30] (ALP)

ALP is a membrane bound glycoprotein enzyme, produced by many tissues, especially bone, liver, intestine, placenta and is excreted into the bile. Elevation in activity of the enzyme can thus be found in diseases of bone, liver and in pregnancy. ALP levels in serum are abnormally high in biliary obstruction, slight to moderate increase in seen in parenchymal liver diseases such as in hepatitis and cirrhosis and in metastatic liver disease. Its normal serum level is 20-100 U/L.

Principle

The substrate, p-nitrophenyl phosphate (PNPP) is hydrolysed by ALP to p-nitrophenol (PNP)(colourless) and phosphoric acid, PNPP is colourless in acid or alkaline medium while PNPis yellow in the alkaline medium and colourless in acid mediumand the concentration is determined by measuring the absorbance at 405 nm.

ALP

P-nitrophenyl phosphate $+H_2O$----------> P-nitrophenol (colourless) + H_3PO_4 (yellow)

Increased alkaline phosphatase activity may be related to hepatobiliary and bone diseases. Very high alkaline phosphatases activity in serum is seen in patient with bone cancer and marked increased also occur in obstructive jaundice and biliary cirrhosis. Moderated elevations havebeen noted in case of Hodgkin's disease, congestive heart failure, infective hepatitis and abdominal problems.

2. Bile pigments

Billirubin or hematoidin is the yellow breakdown product of normal heme catabolism. Heme is found in haemoglobin, a principle component of red blood cells. Bilirubin excreted in bile and urine, and elevated levels may indicate certain diseases. It is responsible for the yellow colour of brusis, urine and the yellow discolouration in jaundice. Bilirubin in serum would only react with diazo reagent in the presence of alcohol, after the proteins had been removed by precipitation. Normally 0.25mg/dl of conjugated bilirubin is present in the blood of an adult. Bilirubin rises in disease of hepatocytes, obstruction to biliary excretion into duodenum in haemolysis, and defects of hepatic uptake and conjugation of bilirubin pigment such as Gilbert's disease. Estimation of bilirubin is one of the better liver

function tests because the liver must take bilirubin away from the albumin to which it is bound in the circulation, conjugate it and excrete it into the bile-a truly complex series of reaction.

Estimation of serum total bilirubin[31,32,33] (TB)

The serum bilirubin level is one of the best tests of liver function tests of liver function. Bilirubin is the metabolic product of the breakdown of the haeme derived from senescent redblood cells .each day about 7.5g of haemoglobin is catabolised with the corresponding production of 250 mg bilirubin. Normally, 025mg/dl of conjugated bilirubin present in the blood of an adult. Bilirubin level rises in diseases of hepatocytes, obstruction to billiary excretion into duodenum, in haemolysis and defects of hepatic uptake and conjugation of bilirubin treatment such as Gilbert's disease.

Principle

Bilirubin reacts with diazotized sulphanilic acid to form an azo compound, the colour of which is measured at 546 nmand is proportional to the concentration of bilirubin. The stability of final colour of reaction of mixture is 10 minutes for total bilirubin and 5 minutes for direct bilirubin.

3. Estimation of serum total protein[34,35] (TP)

Principle

The peptide bond of protein react with CU^{2+} ions in alkaline solution to form a blue violet complex (Biuret reaction), each copper ion complexing with 5 or 6 peptide bonds. Tarterate is added as stabilizer while iodine is used to prevent auto reduction of alkaline copper complex. The color formed is proportional to the protein concentration and is measured at 546 nm.

LITERATURE REVIEW

Sowjanya G et al,. 2013[1]

Liver diseases are a major problem of worldwide proportions and liver damage is very common since liver has the capacity to detoxicate toxic substances. In this review some of the plants with their extract studied for protective effect in liver diseases were summerised. The varios isolated compounds studied herein are Andrographolide, neoandrographolide, bacoside-A, colchicine, populnin, naringenin, echinacoside, kolaviron, ternatin, indigtona, rubiadin, bacicalein, baicalin, wogonin, punicalagin, punicalin for their hepatoprotective activity. There are several chemicalshave been known to induce heptotoxicity by producing reactive species which cause depletion in tissue thiol, lipid peroxidation, plasma membrane damage like carbon tetrachloride, paracetomol, thioacetamide, antituberculer drugs, D-galactosamine, liposachharide and arsenic etc.

Schandalik R et al,. 1992[2]

Liver diseases are among the most serious ailment and can be classified as acute or chronic hepatitis (inflammatory liver disease), hepatosis (non inflammatory diseases) and cirrhosis (degeneration disorders resulting in fibrosis of the liver). Beside expensive and ineffectual modern therapeutic agents like steroids and chemotherapy, south east Asian countries like India and China have an edge in treating hepatic disorders by means of their native botanicals. No doubt, the botanicals need to follow proper standardization and quality control modus operandi before their therapeutic usage in human ailments but the applicability in this regard has shown exceptional success. This review is focused on important botanicals standardized for chemical markers, which have shown promising results as hepatoptotective agents.

Sanjay Kumar Jain et al,. 2013[3,4,5,]

Medicinal practitioners have prescribed Ayurveda and drug from herbal origin as a system of medicine in India over centuries. Popularity of herbal is

increasing globally. More efforts need to be directed towards methodological scientific evaluation for their safety and efficacy by subjecting to vigorous preclinical studies followed by clinical trials to unravel the mysteries hidden in plants. Hepatic disease stand as one of the foremost health troubles worldwide with liver cirrhosis and drug induced liver injury accounting 9th leading cause of death in western and developing countries. In this review article attempt has been made to compile reported hepatoprotective plants from India and aboard and may be useful to health professional's scientists' scholar working in the field of pharmacology, therapeutics and pharmacognosy to develop evidence based alternative medicine to cure different kinds of liver disease in man and animals.

Sowjanya et al,. 2013[8,9]

In this review some of the plants with their extract studied for protective effect in liver diseases were summerised. The varios isolated compounds studied herein are Andrographolide, neoandrographolide, bacoside-A, colchicine, populnin, naringenin, echinacoside, kolaviron, ternatin, indigtona, rubiadin, bacicalein, baicalin, wogonin, punicalagin, punicalin for their hepatoprotective activity. There are several chemicals have been known to induce heptotoxicity by producing reactive species which cause depletion in tissue thiol, lipid peroxidation, plasma membrane damage like carbontetrachloride, paracetomol, thioacetamide, antituberculer drugs, D-galactosamine, liposachharide and arsenic etc.

Rachmilewitz et al,. 1950[10,11,12]

Paracetamol toxicity is due to the formation of a toxic metabolite (*N*-acetyl-*p*-benzoquinoneimine) when it is metabolized by cytochrome P450s, which causes oxidative stress and glutathione depletion.

Rage N et al,. 1959[13,]

The hepatotoxic effects of carbon tetrachloride are largely due to its active metabolite, the trichloromethyl radical. These activated radicals bind covalently

to the macromolecules and induce lipid peroxidative degradation of biomembranes, leading to liver damage.

M. B. Shankar et al,. 2005[15]

The hepatoprotective activity of a new benzopyrone derivative (TP) isolated from the alcoholic extract of aerial parts of *Tephrosia purpurea.* Hepatoprotective activity of TP and the alcoholic extract was evaluated using carbon tetrachloride, paracetamol and rifampicin as toxicants. The potency of TP (100 mg/kg) and the alcoholic extract of aerial parts (200 mg/kg), was compared with silymarin (100 mg/kg). The SGOT, SGPT, total and direct bilirubin levels of blood were measured spectrophotometrically Alcoholic extract of *T. purpurea* and TP have shown significant hepatoprotective activity in rats against all the toxicants that were studied.

Saraf S et al,. 1992[16]

It produces hepatotoxicity with extensive central necrosis, fatty metamorphosis, hepatic cell degeneration and necrosis either by inhalation or by subcutaneous administration (0.4-1.5ml/kg

Kiso Y et al 1983[17]

D-galactosamine (800mg/kg i.p.) induces acute hepatotoxocity after 48 hrs of administration with diffused necrosis and steatosis in male wister rats.

Thripati et al,. 1991[20]

Hepatoprotective activity of Picroliv against alcohol-carbon tetrachloride induced damage in rats.

Gulati, R et al,. 1991[21]

Hepatoprotective activity of Boerhaavia diffusa Linn. Against country made liquor induced toxicity in albino rats fed on controlled calorie diet for 7 days .

Goodell et al,. 1944[22]

Thioacetamide (100mg/kg s.c.) induces acute hepatic damage after 48 hrs of administration by causing sinusoidal congestion and hydropic swelling with increased mitosis.

Reitman S et al,. 1957[28]

After 48 h of paracetamol and carbon tetrachloride and 24 h of ethanol administration, the blood was obtained from animals by puncturing the retro orbital plexus. The blood samples were allowed to clot for 45 min at room

temperature. The serum was separated by centrifugation at 2500 rpm at 30°C for 15 min and utilized for the estimation of various biochemical parameters including SGOT and SGPT, ALP, serum bilirubin, serum protein, and serum cholesterol. After the collection of blood samples, the animals were sacrifi ced under deep ether anesthesia and their livers were excised immediately and washed with ice cold saline, and a 10% homogenate was prepared in a phosphate buffer (pH 7.0). The homogenate was centrifuged at 3000 rpm for 15 min at 4°C and the supernatant was used for the estimation of glutathione and lipid peroxidation.

***Balakrishnan L et al,. 2013*[32]**

Significant hepatoprotective effects were obtained against liver damage induced by all the toxins, as evident from changed biochemical parameters like serum transaminases (SGOT and SGPT), alkaline phosphate (ALP), total bilirubin, total protein, and total cholesterol. The results indicate that CCF and PDF could be useful in preventing chemically induced acute liver injury**.**

***Chakraborty et al,. 2011*[49,50,51]**

The effect of the warm aqueous extract of *Lawsonia inermis* leaf on Carbon tetrachloride (CCl4) induced liver damage in healthy Wister albino rats was studied. The results showed that significant hepatoprotective effects were obtained against liver damage induced by CCl4 as evidenced by decreased levels of serum enzymes, glutamate pyruvate transaminase (SGPT), glutamate oxaloacetate transaminase (SGOT), serum alkaline phosphatase (SAKP), serum bilirubin (SB). The extracts also showed significant antilipid peroxidant effects *in vitro*, besides exhibiting significant activity in quenching 1, 1-diphenyl -2-picryl hydrazyl (DPPH) radical indicating its potent antioxidant effects.

***Chaudhary et al,. 2010*[52]**

Lawsonia inermis L. is a much branched glabrous shrub or small tree, cultivated for its leaves although stem bark, roots, flowers and seeds have also been used in traditional medicine. The plant is reported to contain carbohydrates, proteins, flavonoids, tannins and phenolic compounds, alkaloids, terpenoids, quinones, coumarins, xanthones and fatty acids. The plant has been reported to have analgesic, hypoglycemic, hepatoprotective, immunostimulant, anti-inflammatory, antibacterial, antimicrobial, antifungal, antiviral, antiparasitic, antitrypanosomal, antidermatophytic, antioxidant, antifertility, tuberculostatic and anticancer properties. It is now considered as a valuable source of unique natural products for development of medicines against various diseases and also for the development of industrial products. This review gives a bird's eye view mainly on the pharmacognostic characteristics, traditional uses, phytochemistry and pharmacological actions of the plant.

Chang H et al,. 1982[53]

Aqueous extract of leaves of *L. inermis* was found to inhibit sickling and to increase the oxygen affinity of HbSS blood.

Dasgupta T et al,. 2003[54]

Modulator effect of 80 % ethanol extract of leaves of henna on drug metabolising phase I and phase II enzymes, antioxidant enzymes, lipid peroxidation in the liver of Swiss Albino mice. The hepatic glutathione S-transferase and DTdiaphorase specific activities were elevated above basal level by *L. inermis* extract treatment. With reference to antioxidant enzyme the investigated doses were effective in increasing the hepatic glutathione reductase (GR), superoxide dismutase (SOD) and catalase activities significantly at both the dose levels. Reduced glutathione (GSH) measured as non-protein sulphydryl was found to be significantly elevated in liver. Among the extrahepatic organs examined (forestomach, kidney and lung) glutathione S-transferase and DTdiaphorase level were increased in a dose independent manner.

Aguwa CN et al,. 1987[55]

Methanol extract of roots of *L. inermis* was most effective in inducing abortion in mice, rats and guinea pig. The effect apparently was dosage dependent. The results of the whole animal experiments support the methanol extract effectiveness as an abortant due to its maternal and foetal toxic effects.

Yogisha S et al,. 2002[56]

The ethanol extract of *L. inermis* L. leaves and lawsone tested for trypsin inhibitory activity showed an IC50 value of 64.87 and 48.6µg/ml, respectively.

Khan M et al,. 1991[57]

The ethanol soluble fraction of *L. inermis* fruits displayedhighly potent activity against Sembiki forest virus (SFV) in swiss mice and chick embryo models exhibiting 100 to 65 % activities after 10 to 25 days of virus challenge.

Endrini S et al,. 2007[58]

Chloroform extract of leaves of *L. inermis* displayed the cytotoxic effects against liver (HepG2) and Human breast (MCF-7) with IC50 values of 0.3 and 24.85µg/ml by microculture tetrazolium salt assay (MTT).

Syamsudin et al,. 2008[59] Activity of Ethanol (70%) extract showed significant Hypoglycaemic and hypolipidaemic activities in alloxan induced diabetic mice after oral administration.

Endrini et al., 2007[60]

Chloroform extract of leaves of *Lawsonia inemris* had shown the highest activity (87.6%) followed by α-tocopherol (62.5%) by using FTC method and based on TBA method significant activity (55.7 %) compared to α-tocopherol (44.4%).

Selvanayagi R et al,. 2012[61,62]

The hepatoprotective activity of aqueous extract of lawsonia inermis against paracetamol induced hepatotoxicity in rats. The aqueous extract of lawsonia inermis administered orally to the animals with hepatotoxicity induced by paracetamol. Silymarin was given as a reference standard. The plant aqueous extract was effective in protecting the liver against the injury induced by paracetamol induced toxicity. This was evident from significant reduction in serum enzymes ALT, AST, ALP, ACP, protein and bilirubin.

***Gupta A et al,. 1986*[63]**

Isoplumbagin and lawsaritol, isolated from stem bark and root of *L. inermis* L. showed anti- inflammatory activity against Carrageenan induced paw oedema in rats. The compounds phenylbutazone, isoplumbagin and lawsaritol at the oral dose of 100 mg/kg exhibited and 40 percent inhibition in comparison with controls. Isoplumbagin showed significant anti- inflammatory activity similar to that of phenylbutazone. Butanol and chloroform fractions showed more potent anti-inflammatory, analgesic andantipyretic effects than aqueous fraction of crude ethanol extract of *L. inermis* in a dose dependent manner. Leaves showed significant anti-inflammatory effect with some active principles.

Bhanwa *et al, 2000* [64]

Studied the effect of aqueous leaf extract of *azadirachta indica* in paracetamol-induced hepatotoxicity in rats. The liver damage due to paracetamol administration resulted in elevation in the activities of serum transaminases and gamma glutamyl transpeptidase (GGT). The extract of A.indica was found to be effective in reducing paracetamol-induced liver necrosis as evidenced by histopathological studies.

***Aniya et al., 2000*[65]**

Aqueous extract of Artemisia campestris exhibited hepatoprotective and anti oxidant activities (in vivo and in vitro). The extract showed scavenging action of 1,1,diphenyl picrylhydrazyl, hydroxyl and superoxide anion radicals.

Pretreatment (intraperitoneal/oral) with the extract significantly reduced the CCL_4 evoked elevation of serum transaminases in mice. The authers suggested thet the proactive action of A.campestris extract may be due to scavenging action of the plant for free radicals formed by CCL_4 treatment.

Ahmed et al., 2000[66]

Pre-treatment of rats with the 50% ethanol extract of the bark of lawsonia alba showed hepatoprotective activity against CCL_4 induced oxidative stress. The prptective activity of the extract was shown by the reduction in the activities of serum transaminases and lactate dehydrogenase (LDH) in the rats against in the activities of the enzymes when challenged with CCL_4. moreover, the plant extract prevented CCL_4 induced oxidative stress by maintaining the levels of reduced glutathione, its metabolising enzymes and simultaneously inhibiting the production of free radicals.

Desmarchelier et al., 1999[67]

The in vitro antioxidant and free radical scavenging properties of the park extract of anadenanthera macrocarpa, astronium urundeuva, mimosa verrucosa and sideroxylon obtusifolium were studied using different bioassays. All the extracts (aqueous and methanolic extract) were active.

Bhaka et al., 1999[68]

Evaluated the antihepatotoxic of n-heptane extract of cassia fistula leaves in CCL_4 : liquid paraffin (1:1) treated rats.Biochemical and histopathological investigation indicated that the extract of C.fistula (400 mg/kg body weight) has hepatoprotective effect.

Jafri et al., 1999[69]

Biochemical and histopathological studies on the effect of the leaves of C.occidentalis (aqueous-ethanolic extract) on paracetamol and ethanol intoxication in rats revealed its hepatoprotective activity.

Lalitha and selvam., 1999[70]

Turmeric antioxitant protein isolated from the aqueous extract of turmeric (Curcuma longa) has been found to exhibit hepatoprotection in CCL_4-treated rats. The studies shown the decrease in the activities of antioxidant enzymes in liver due to CCL_4 intoxication was nearly normolized on treatment with the protein. The authers suggest that the protection exhibited by the protein may be due to the stabilization of the oxidative stress induced changes.

Germane et al., 1999[71] The studied effect of Mitracarpus scaber (decoction of aerial parts) on CCL_4-induced acute liver damage (in vivo and in vitro) in rats. In vivo results showed that pre-treatment with M.scaber reduced the elevated activities of serum GOT and GPT due to CCL_4 treatment. In vitro results indicated that addition of M.scaber extracts to the culture medium reduced the CCL_4-evoked elevation in the activities of serum GOT and GPT. In vitro study also revealed the free radical scavenging properties of M.scaber.

Manjunath BK et al,. 2008[72]

Aqueous and ethanol extract of leaf of *Vitex trifolia* was investigated for hepatoprotective activity against carbon tetrachloride induced liver damage. To assess the hepatoprotective activity of the extracts, various biochemical parameters viz. total bilirubin, total protein, alanine transaminase, aspartate transaminase and alkaline phosphatase activities were determined. Results of the serum biochemical estimations revealed significant reduction in total bilirubin and serum marker enzymes and increase in total protein in the animals treated with ethanol and aqueous extracts. However significant rise in these serum enzymes and decrease in total protein level was noticed in CC14 treated

group indicating the hepatic damage. The hepatoprotective activity also supported by histological studies of liver tissue. Histology of the liver tissue treated with ethanol and aqueous extracts showed normal hepatic architecture with few fatty lobules. Hence the present study revealed that Vitex trifolia could afford significant protection against CCl_4 induced hepatocellular injury.

Patel JA et al,. 2009[73,74,75,76,]

Piper longum Linn. (Piperaceae) (Fruits and roots powder) is given with boiled milk in the Indian traditional system of medicine for the treatment of liver ailments and jaundice. However, the biochemical basis and mechanism of hepatoprotective action of *Piper longum* milk extract, is not scientifically studied. Thus, the present study was designed to investigate the hepatoprotective activity of Piper longum milk extract. Carbon tetrachloride (CCl_4) was used as a hepatotoxin at a dose of 0.5ml/kg p. o. with olive oil (1:1) thrice a week for 21 days to produce the chronic reversible type of liver necrosis. Following treatment with *Piper longum* milk extract (200 mg/day p. o. for 21 days), a significant hepatoprotective effect was observed in CCl_4 induced hepatic damage as evident from decreased level of serum enzymes, total bilirubin and direct bilirubin. The hepatoprotective effect of Piper longum is comparable to the standard drug silymarin (25 mg/kg/day p. o. for 21 days).

Harish Chander Dutt et al,. 2012[77,78,79,80]

The hepatoprotective effect of aqueous ethanol extract of *Z. officinale* against acetaminophen induced acute toxicity is mediated either by preventing the decline of hepatic antioxidant status or due to its direct radical scavenging capacity. Liver damage by CCl_4 can also be prevented by the roots of the plant species. Ginger oleoresin when administered orally significantly lowered serum and hepatic cholesterol and increased faecal cholesterol excretion.

Abdel-Tawab H. Mossa et al,. 2012[80,81,82]

To evaluated the adverse effect of exposure to diazinon and paracetamol and their combination on male rats. Significantly, decreased of body weights were observed in all treated groups, while significant increase in relative liver weight were recorded in DIA and DIA+PARA treated groups compared to control rats. Liver dysfunction enzymes (ALT, AST, ALP) were increased in DIA and DIA+PARA treated group. DIA and DIA+PARA treated groups are significantly changes in the biochemical and histopathalogical changesand decreases DNA content in the rat liver cells.

Bhaskar VH et al,. 2010[83,84]

Paracetamol toxicity is due to the formation of a toxic metabolite (*N*-acetyl-*p*-benzoquinoneimine) when it is metabolized by cytochrome P450s, which causes oxidative stress and glutathione depletion.

M. B. Shankar et al,. 2005

The hepatoprotective activity of a new benzopyrone derivative (TP) isolated from the alcoholic extract of aerial parts of *Tephrosia purpurea.* Hepatoprotective activity of TP and the alcoholic extract was evaluated using carbon tetrachloride, paracetamol and rifampicin as toxicants. The potency of TP (100 mg/kg) and the alcoholic extract of aerial parts (200 mg/kg), was compared with silymarin (100 mg/kg). The SGOT, SGPT, total and direct bilirubin levels of blood were measured spectrophotometrically Alcoholic extract of *T. purpurea* and TP have shown significant hepatoprotective activity in rats against all the toxicants that were studied.

Galighor AE et al,. 1971

A Small piece of liver were fixed in 10% neutral buffered formalin and subsequently embedded in paraffin. A transverse section of 5 μm was cut from each sample and stained with haematoxylin and eosin. Histopathological assessment (light microscopy) was performed on randomized sections of liver.

AIM OF STUDY

Liver disease are the biggest threat to the world which is characterised with impaired metabolic secretary function of liver clinically as jaundice, cirrhosis, hepatitis, liver cancer and ultimately liver failure. About 20,000 deaths founds every year due to liver disorders. A common chronic disease known as liver fibrosis may lead to end-stage cirrhosis and liver cancer. Hepatocellulor carcinoma is the one of the ten most common tumers in the world with over 2,50,000 new cases each year.

In the treatment of liver diseases in modern medicine cartico steroids and immunosuppressants are commonly used to treat to liver disease in allopathic form of medicine. But these drugs are associated with adverse effects such as immunosuppression and bone marrow depression. Further, the success rate of treating liver diseases is disappointing. Attempts are being made globally to get scientific evidence for this traditionally reported herbal drugs.

Lawsonia inermis L. belonging to the family of lythraceae. A shrub commonly known as mehendi and henna. Mainly the aim of the study was evaluate the hepatoprotective activity as evidence from the literature review.

The *lawsonia inermis* is traditionally used in india for the treatment of jaundice, enlargement of liver and spleen. It has been reported to possesses the anti-inflammatory, anti-microbial and anti-cancer activity. Because the one of the major active component of the leaves of *lawsonia inermis* are flavanoids. The hepatoprotective activity of warm aqueous extract of leaves of *lawsonia inermis* has been reported. But detail study of hepatoprotectivity has not done. The present study has been undertaken with aim to determine the hepatoprotective activity of ethanolic extract of *lawsinia inermis* in animal model by estimating biochemical and histopathalogical parameters.

PLANT PROFILE

Synonym: lawsonia alba lam, lawsonia spinoza

CLASSIFICATION[36]

Kingdom : Plantae

Sub kingdom : Tracheobionta

Super division : Spermotophyta

Division :Magnoliophyta

Class : Magnoliopsida

Subclass : Rosidae

Order :Myrtales

Family :Lythraceae

Genus : Lawsonia L.

Species :Lawsonia inermis L.

VERNACULAR NAMES[37,38,39]

English : Henna

Hindi : Mehndi

Kannada : Mayilanchi

Malayalam : Mailanchi

Sanskrit **:** Rakigarbha,Mendika

Tamil **:** Maruthani

Telugu: Goranta

DISTRIBUTION[39,40]

lawsonia inermis Linn native to north Africa and south east asia, and often cultivated as an ornamental plant throughout india, Persia, and along the African coast of the Mediterranean sea.

DESCRIPTION[41,42]

Lawsonia inermis Linn (Lythraceae). is aglabrous branched shrub or small tree (2 to 6 m in height). Leaves are small, opposite, entire margin elliptical to broadly lanceolate, sub-sessile, about 1.5 to 5 cm long, 0.5 to 2 cm wide, greenish brown to dull green, petiole short and glabrous acute or obtuse apex with tapering base. New branches are green in colour and quadrangular, turn red with age. Young barks are greyish brown, older plants have spine tipped branchlets. Inflorescence has large pyramid shape cyme. Flowers are small, numerous, aromatic, white or red with four crumbled petals. Calyx has 0.2 cm tube and 0.3 cm spread lobes. The fruits are small, brown globose capsule, opening irregularly and split into four section with a permanent style. Seeds have typical, pyramidal, hard and thick seed coat with brownish coloration.

CHEMICAL CONSTITUENTS[43,44,45]

The chemical constituents isolated from leaves of ***l. inermis*** are phenolic compounds, phytosterols, coumarin, flavanoids and essential oils are present.

Napthoquinone derivatives: Lawsone (2-hydroxy 1,4-napthoquinone) 1,3-dihydroxy naphthalene, 1,4-napthoquinone, 1,2-dihydroxy-4-glucosylnapthalene.

Phenolic compounds: lawsoniaside (1,3,4-trihydroxynapthalene 1,4-dibeta-D-glucopyronoside), lalioside (2,3,4,6-tetrahydroxyacetoxy-2beta-D-glucopyrasonide), lawsoniaside B (3-(4-0-a-D-glucopyranosyl-3,5-dimethoxy) phenyl-2E-propenol), syringioside, daphneside, daphnorin, agrimonolide 6-O-Beta-D-glucopyrasonide, (+)-pinoresinol di-O-beta-D-glucopyranoside, syringaresinol di-O-D-glucopyranoside, isoscutellarin.

Phytosterols: lawsaritol (24beta-ethycholest-4-en-3beta-ol) stigmosterol and β-sitosterol.

Flavonoids: Apigenin-7-glucoside, apigenin-4-glycoside, luteolin-7-glucoside, luteolin-3-glucoside.

Essential oil: -(z)-2-hexenol, linalool, α ionone, α-terpeneol, β-ionone, terpinolene, δ-3-carene and γ-terpineol.

PARTS USED[46]

Fresh leaves.

TRADITIONAL USES[47,48]

Lawsonia inermis is awell known ethnomedical plant used cosmetically and medicinally for over 9,000 years. Its use in the Indian traditional folk medicines is well documented. Below that table was indicates the use of different parts of *l. inermis* in traditional system of medicine.

Plant parts	Traditional uses (as/in)
	Bitter, astringent, acrid, diuretic, emetic, edema, expectorant, adonyne, anti-inflammatory, constipating, depurative, **liver tonic,** haematinic,

Leaves	styptic, febrifuge, trichogenuos, wound, ulcers, srangury, cough, bronchitis, burning sensation, cephalalgia, hemicarnis, lumbago, rheumatalgia, inflammations, diarrhoea, dysentery, leprosy, leucoderma, scabies, boils, **hepatopathy,** splenopathy, anemia, haemorrhages, hemoptysis, fever, opthalmia, amenorrhoea, falling of hair, greyness of hair, **jaundice.**
Root	Bitter, depurative, diuretic, emmenagogue, abortifacient, burning sensation, leprosy, skin disease, amenorrhoea, dysmenorrhoea and premature greying of hair.
Flowers	Cardiotonic, refrigent, soporific, febrifuge, tonic, cephalalgia, burning sensation ,cardiopathy, amentia, insomnia, fever.
Seeds	Antipyretic, intellect promoting, constipating, intermittent fevers, insanity, amentia, diarrhoea, dysentery and gastropathy.

PLAN OF WORK

Objectives:

The main objective of the study was evaluate the hepatoprotective activity of ethanolic extract of the leaves of *Lawsonia inermis linn* in validated experimental animals.

Step: 1

Idendification and authentification of plant *Lawsonia inermis linn* by renowed taxonomist.

Step: 2

Collection of leaves of *Lawsonia inermis linn* and its shade drying.

Step: 3

Extraction of the leaves of *Lawsonia inermis* using solvent of ethanol.

Step: 4

Evaluations of phytochemical constituents

Step: 5

Acute toxicity studies as per OECD guidelines.

Step: 6

Study the hepatoprotective activity of ethanolic extract of *Lawsonia inermis* using paracetamol induced hepatotoxicity model.

Step: 7

Evaluations of following parameters
a)Biochemical parameters

b)Histopathalogical studies.

MATERIALS AND METHODS

COLLECTION OF PLANT

The leaves of *lawsonia inermis linn* were collected in June 2013, from Telungupalayam, Coimbatore District, Tamilnadu, India. The plant was taxonomically identified by the botanist Dr.M.Palanisamy scientist 'C' incharge, Head of office, Botanical survey of India , Southern Regional Centre, Coimbatore – 641003..

EXTRACTION OF PLANT

The collected leaves are washed with tap water and shade dried for 1 week. One week after the drying, materials were converted in to coarse powder by using mixer. Taken 100g of coarse powder of plants, mixed with 500ml of 70% ethanol in round bottom flask, which was kept under the process reflux for 72 hours by using Soxhlet apparatus. The extracts are dried under reduced pressure and stored in desiccators. The residue (50 g) were collected and used for the present investigation.

CHEMICALS

- ✓ Ethanol 70%.
- ✓ chloroform.
- ✓ Distilled water.
- ✓ Formalin solution.
- ✓ Carboxyl methyl cellulose.

DRUGS [32]

- ✓ Paracetamol.
- ✓ Silymarin (100mg/kg)

SOLVENTS

Ethyl alcohol, Water, CMC (carboxyl methyl cellulose)

QUALITATIVE PHYTOCHEMICAL SCREENING

The following tests were carried out on the standardized herbal extract to detect the various phytoconstituents present in the plant extract.

1. TEST FOR CARBOHYDRATES

A small quqntity of the extract was dissolved in the distilled water and filtered. The fitrate was subjected to the following test for carbohydrate.

a) Molisch's Test

To the filtrate few drops of alcoholic α-napthol was added and 2ml of concentrated sulphuric acid was added slowly through the sides of the test tube. No purple color of ring was formed, which indicates absence of carbohydrate.

b) Fehling's Test

A small portion of the extract was treated with fehlings solution A&B then heated on water bath. No brick red colored precipitated was formed, which indicates absence of carbohydrate.

c) Barfoed's Test

A small portion of the extract treated with barfoeds reagent. No red colored precipitated was formed, which indicates absence of carbohydrate.

2. TEST FOR STARCH

A small portion of the extract was treated with dilute iodine solution. No blue color was formed, which indicates absence of starch.

3. TEST FOR AMINO ACIDS AND PROTEINS

A small quantity of the extract was dissolved in the disstiled water and filtered. The filtrate was subjected to millions and biured test and ninhydrin test.

a) Million's Test

The filtrate is treated with millons reagent. No white precipitated was produce, which indicates absence of protein and amino acids in the filtrate.

b) **Biuret Test**

To the filtrate equal volume of 5% w/v of NaOH and four drops of 1%w/v $CuSO_4$ solution were added. No purple or red color was formed, so it indicates absence of protein.

c) **Ninhydrin Test**

The filtrate is treated with ninhydrin reagent. No purple color was formed indicating absence of protein.

4. TEST FOR PHENOLIC COMPOUNDS

Small quantities of the extracts were taken separately in water and test for the presence of phenolic compounds and tannins was carried out with the following reagents.

a) Ferric Chloride Test

the filtrate was treated with 5% of ferric chloride solution. Black color of precipitate is formed, which indicates presence of and tannins phenolic compounds.

b) Lead Acetate Test

few ml of filtrate is treated with lead acetate solution. White precipitate was produced, it indicate presence of phenolic compounds.

c) Gelatin Test

To the filtrate add 1ml of gelatin solution. White colour precipitate was seen indicate presence of tannins.

5. TEST FOR PHYTOSTEROL

The extract was refluxed with solution of alcoholic potassium hydroxide till complete saponification has taken place. The mixture was diluted and extracted with ether. The ether layer was evaporated and the residue was tested for the presence of phytosterol.

a) Libermann Burchard Test

The residue was dissolved in few drops of dil. Acetic acid; 3 ml of acetic anhydride was added followed by few drops of Con. Sulphuric acid. Bluish green color shows the absence of phytosterol.

6. TESTS FOR ESSENTIAL OILS

Spot Test

A small quantities of various extracts were separately pressed between two filter papers. Appearance of oil stain on the paper indicates the presence of fixed oil. Few drops of 0.5N alcoholic potassium hydroxide were added to small quantity of various extracts along with a drop of phenolphthalein. The

mixture was heated on a water bath for 1-2 hours. Formation of soap or partial neutralization of alkali indicates the presence of oils.

7. TEST FOR ALKALOIDS

A small portion of the solvent free alcohol and aqueous extracts were stirred separately with few drops of dilute hydrochloric acid and filtered. The filtrate was tested with various reagents for the presence of alkaloids.

a) Mayer's Test

To the small amount of the filtrate was treated with mayer's reagent. No white color of precipitated was formed, indicatings absence alkaloids.

b) Dragendroff's Test

To the small amount of the filtrate was treated with Dragendroff's reagent. No orange or red color was formed, indicating absence of alkaloids.

c) Wagner's Test

To a small amount of the filtrate is treated with Wagner's reagent. No brown color precipitate formed, indicating absence of alkaloids.

d) Harger's Test

To the small amount of the filtrate is treated with few drops of Harger's reagent. No yellow crystalline precipitate formed, indicating absence of alkaloids.

8. **TESTS FOR GLYCOSIDES**

a) Legal's Test

To the hydrolysate I ml of pyridine and few drops of sodium nitropruside solutions were added and then it was made alkaline with sodium hydroxide solution. Appearance of pink to red colour shows the presence of glycosides.

b) Bortanger's Test:

Hydrolysate was treated with choloroform and then the chloroform layer was separated. To this equal quantity of dilute ammonia solution added. Ammonia layer acquires pink color, showing the presence of glycosides.

9. TEST FOR FLVAVONOIDS

The extract was dissolved in ethanol and then subjected to following test.

a) Ferric Chloride Test

To a small quqntity of the ethanol extract with few drops of neutral ferric chloride solution was added. Blackish red color was observed, showing the presence of flavonoids.

b) Shinoda's Test

To a Small quantities of the extract were dissolved in alcohol, to them piece of magnesium followed by Conc. Hydrochloric acid drop wise added and heated. Appearance of magenta color shows the presence of flavonoids.

c) Concentrating Sulphuric Acid test

To a small quantity of the extract was treated with concentrated H_2SO_4. Yellow orange color, presence of flavanoids.

d) **Lead acetate test**

To a small quantity of the extract was treated with 10% lead acetate solution. to form yellow color precipitate indicating presence of flavanoids.

10. TEST FOR COUMARINS

To a small quantity of the extract were dissolved in alcohol and ferric chloride solution, shows green color, indicating presence of coumarins.

ACUTE ORAL TOXICITY STUDIES

PROCEDURE[80]

Preparation of animals

The animals are randomly selected, marked to permit individual identification, and kept in their cages for at least 5 days prior to dosing to allow for acclimatisation to the laboratory conditions.

Preparation of doses

In general test substances should be administered in a constant volume over the range of doses to be tested by varying the concentration of the dosing preparation. Where a liquid end product or mixture to be tested however, the use of the undiluted test substances, i.e. at a constant concentration, may be more relevant to the subsequent risk assessment of the substances, and is a requirement of some regulatory authorities. In either case, the maximum dose volume for administration must not be exceeded. The maximum volume of the liquid that can be administrated at one time depends on the size of the test animal. In rodents, the volume should not normally exceed 1ml/100 gm of body weight. However in the case of aqueous solution 2ml/100 gm body weight can be considered. With respect to the formulation the dosing preparation, by use of an aqueous solution/ suspension/ emulsion is recommended wherever possible, followed in order of preference by solution/suspension/emulsion in oil ex. (Corn oil) possible solution in other vehicle. For vehicles other then water the toxicological characteristics of the vehicle should be known. Doses must be prepared shortly prior to administration unless the stability of the preparation over the period during which it will be used is known and shown to be acceptable.

Administration of the dose

The test substance is administered in a single dose by gavages by using a stomach tube or suitable incubation cannula. In the unusual circumstance that a single dose is not possible, the dose may be given in small fraction over period not exceeding 24 hours.

Animal should be fasted prior to dosing (e.g. with rat, food but not water should be withheld over night, with the mouse food but not water should be withheld for 3-4 hours) following the period of fasting, the animal should be weighed and the test substances is administered. After the substance has been administered, food may be withheld for a further 3-4 hours in rats or 1-2 hours in mice. Where a dose is administered infraction over a period it may be necessary to provide the animals with food and water depending on the length of period.

Number of the Animal and Dose Levels

Three animals are used for each step. The dose level to be used as the starting dose is selected from one hour of four fixed levels, 5, 50, 300, 2000 mg/kg body weight. The starting dose level should be that which is most likely to produce mortality in some of the dosed animals.

When available information suggests that mortality is unlikely at the highest starting dose level (2000 mg/kg body weight), then a limit test should be conducted. When there is no information on a substance to be tested, for animal welfare reasons it recommended using the starting dose of 300 mg/kg body weight.

The time interval between treatment groups is determined by the onset, duration, and severity of toxic signs. Treatment of animals at the next dose should be delayed until one is confident of survival of the previously dosed animals.

Exceptionally, and only when one justified by specific regularity needs the use of additional upper dose level 5000 mg/kg body weight may be considered. For reason of animal welfare concern, testing of animals in GHS category 5 ranges (2000 – 5000 mg/kg) is discouraged and should only be considered and when there is a strong likelihood that results of such a test have direct relevance for protecting human or animal health or the environment.

Limit test:

The limit test is primarily used in situations where the experimenter has information indicating that the test material is likely to be nontoxic, i.e., having toxicity only above regulatory limit doses. Information about the toxicity of the test material can be gained from knowledge about similar tested compounds or similar tested mixtures are products, taking into consideration the identity and percentage of components known to be toxicological significance.

A limit test at one dose level of 2000 mg/kg body weight may be carried out with six animals (3 animals per step). Exceptionally a limit test at one dose level of 5000 mg/kg may be carried out with 3 animals. If test substance-related mortality is produced, further testing at the next lower level may need to be carried out.

Test report:

- Tabulation of response data and dose level for each animal (i.e. animal showing signs of toxicity including mortality; nature, severity, and duration of side effects);
- Tabulation of body weight and body weight changes;
- Individual weights of animals at the day of dosing, in weekly intervals thereafter, and at the time of death or sacrifice;
- Date and time of death if prior to scheduled sacrifice;
- Time course of onset of signs of toxicity, and whether these were reversible for each animals;
- Necropsy findings and histopathological findings for each animal.

SCREENING METHODS FOR HEPATOPROTECTIVE ACTIVITY

ANIMALS

Studies were carried out using either sex wister albino rats (160-200g) and swiss albino mice (18-25 g).They were obtained from the animal house of agri culture university, trissur, kerala, india. The animals were grouped and housed in polyacrylic cages (38×23×10 cm) with not more than six animals per cage and maintained under standard laboratory condition (temperature 25±2°C), relative humitity (55±5%) with dark and light cycle (12/12 h). They were allowed to standard pellet diet (Amrut feed, Hindustan lever, Bangalore, india) and water ad libitum. The rats were acclimatized to laboratory condition for 10 days before commencement of experiment. The animal studies were approved by the committee for the control and supervision of experiment on animals (CPCSEA) and conducted according to the regulation of Institutional Animal Ethics Committee(IAEC) with project No.M.Pharma/13/10.

EXPERIMENTAL DESIGN[81,82]

PARACETAMOL-INDUCED HEPATOTOXICITY

Group I	-	Normal Control (normal saline 5ml/kg, p.o)
Group II	-	Toxicant (paracetamol 2g/kg, p.o)
Group III	-	Standard drug treated (silymarin 100mg/kg, p.o)
Group IV	-	plant extract (EELI 200mg/kg, p.o)
Group V	-	plant extract (EELI 400mg/kg, p.o)

EXPERIMENT PROCEDURE[83,84]

Paracetamol induced hepatotoxicity model was adobted for the study. The rats were divided into 5 groups of 6 animals each. Group I served as a normal control and received normal saline, 5 ml/kg body weight, daily for 7 days. Group II constituted the hepatotoxicity group and were treated similarly to group I. Group IV and group V received ethanolic extract (200 and 400 mg/kg body weighy per day, respectively) suspended in 0.5% sodium carboxy methyl cellulose for 7 days. Group III received the standard drug, silymarin (100 mg/kg body weight daily) for 7 days. On the 7 th day after 2 hours of respective treatment, paracetamol suspension was given orally, 2 g/kg body weight, to all the rats except those in group I. At the end of the experimental period, the rats were fasted overnight and blood samples were collected from retro-orbital plexus for biochemical studies and animals are sacrificed under anesthesia (pentobarbitone sodium, 45mg/kg).Then liver samples were collected for histopathological studies.

BIO-CHEMICAL STUDIES[88,89,90]

Blood was obtained from all animals by puncturing the retro-orbital plexus. Blood samples were allowed to clot for 45 min at room temperature. Serum was separated by centrifugation for 3000 rpm for 10 minutes to separate the serum. The separated blood serum was analyzed for the biochemical markers of SGPT (ALT), SGOT (AST), ALP, Bilirubin, Total protein levels.

HISTOPATHALOGICAL STUDIES[91]

After the collection of blood samples, animals were sacrificed, liver samples collected, washed with normal saline and were fixed in 10% buffered neutral formalin for 48 hours and then washed with water to remove fixative. The tissue were

fixed in bouins solution for six hours and processed for microtome section and examined in light microscope.

STATISTICAL ANALYSIS

The results were expressed as Mean ± S.D, (N=6). Statistical significant values were determined by one-way analysis (ANOVA) followed by Dunnett's test. $P<0.05$ was considered significant.

RESULTS AND DISCUSSIONS

Table-1

CHEMICAL CONSTITUENTS OF DIFFERENT EXTRACTS OF LEAVES OF *LAWSONIA INERMIS* LINN

Chemical Constituents	Aqueous extract	Non aqueous extract
Carbohydrates	-	-
Glycosides	-	-
Alkaloids	-	-
Flavonoids	+	+
Phenols	+	+
Fixed oils	-	+
Steroids	+	+
Saponins	+	-
Gums & mucilage	-	-
Proteins & free amino acids	-	-
Tannins	+	+
Terpenoids	+	-

(+) → Positive

(-) → Negative

ACUTE ORAL TOXICITY FOR EELI

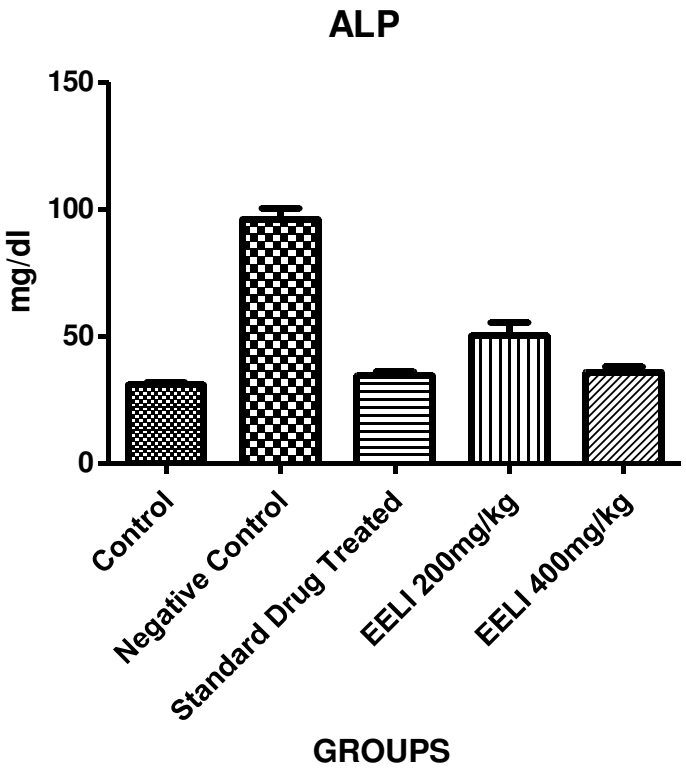
TREATMENT	Mg/kgDOSE	MORTALITY	SEDATION	CONVULSION	URINATION	BODY COLOR CHANGES	LOCOMOTION	EIGHTBODY
EELI	mg/kg5	-	-	-	-	-	-	-
EELI	50 mg/kg	-	-	-	-	-	-	-
EELI	300mg/kg	-	-	-	-	-	-	-

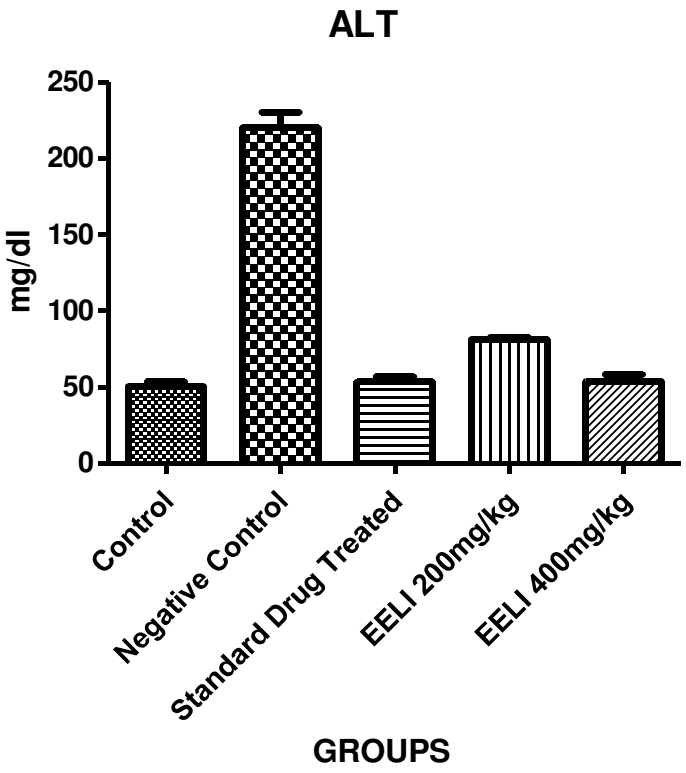
Based on the mortality result of sighting study starting dose in main study is decided and carried out with six animals per dose level (2000mg/kg). Based on the mortality result on 14th day of observation, the doses for *in vivo* study were selected.

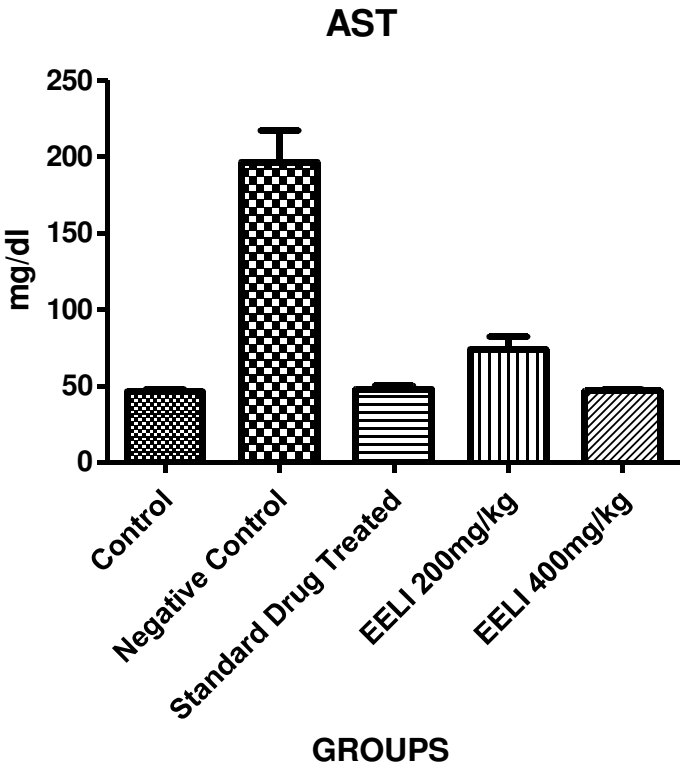
EFFECT OF EELI ON SERUM ENZYMES IN PARACETAMOL INDUCED HEPATOTOXICITY

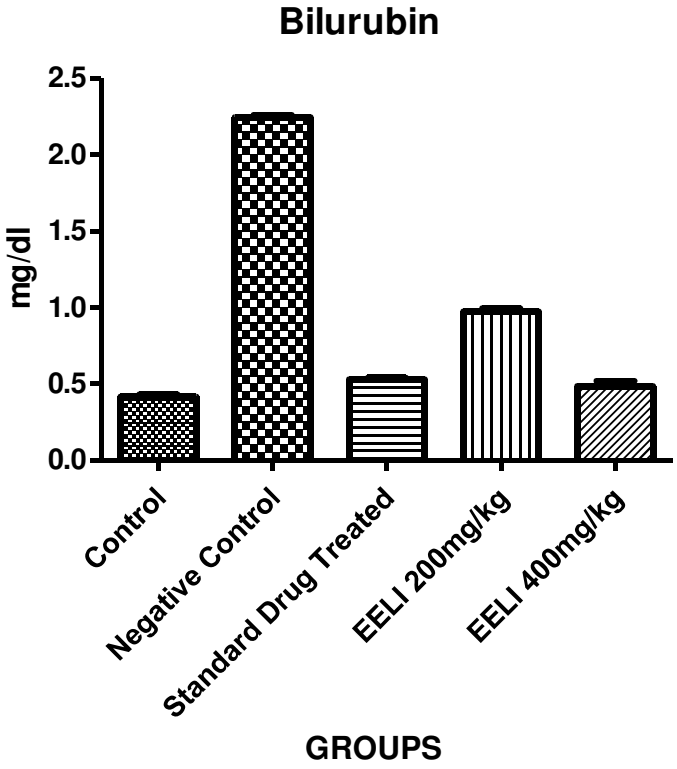
Treatment /group (Mg/kg, P.O)	ALP	ALT	AST	Total bilirubin	Total protein
Control Normal saline (5ml/kg)	31±0.577	50.66± 1.667	46.33 ±0.881	0.416±0.008	5.833± 0.1202
Negative control (PCM 2gm/kg)	96±2.517	220.07 ±5.774	196.66±12.020	2.246±0.008	7.8±0.1528
Standard drug Treated Silimayrin (100mg/kg)	34.66± 0.881	53.66± 1.856	48.33 ±1.202	0.53±0.005	5.366± 0.1202
Plant extract (EELI 200mg/kg)	50.33± 2.963	81.33± 0.881	74.33 ±4.702	0.97±0.012	6.566± 0.1202
Plant extract (EELI 400mg/kg)	36 ± 1.555	54.07± 2.517	0.48± 0.018	0.48±0.018	6.000± 0.0577

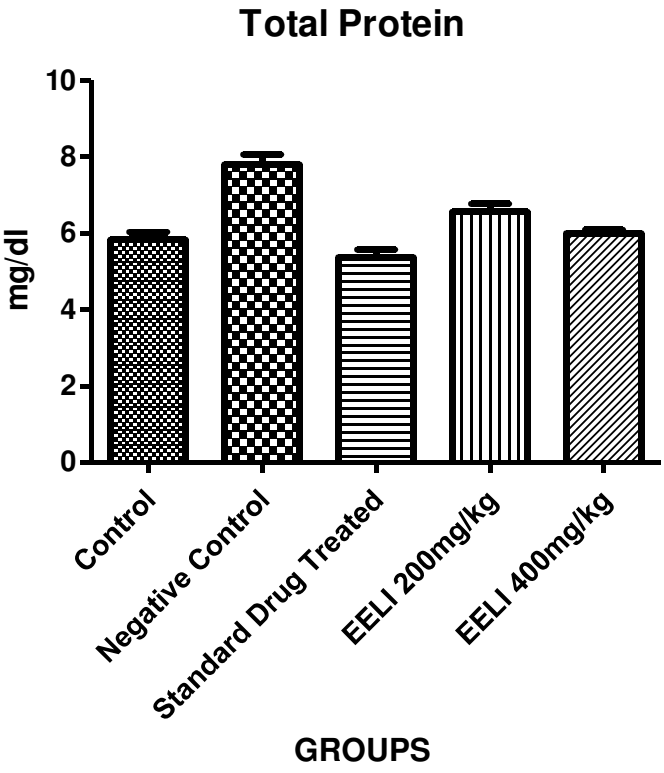
The Paracetamol induced Hepatotoxicity in rats, results have expressed on Table. All the groups of animals were affected in Hepatotoxicity, ethanolic extract of *Lawsonia inermis* treated groups 200 & 400 mg/kg were dose dependent manner decreased ALP & ALT & AST levels (P<0.001)**& (P<0.0001)*** (36±1.555**↓&54.07±2.517**↓),(0.48±0.018↓).When compared with negative control group but positive control have less Hepatotoxicity (P<0.001)**& (P<0.0001)*** When compare to each groups of ethanolic extract of *Lawsonia inermis* 400 mg/kg have high Hepatoprotective activity (0.48±0.018**↓& 6.000±0.0577**↓). When compared with positive control.


ALP
150
100
50
0
mg/dl
Control
Negative Control
Standard Drug Treated
EELI 200mg/kg
EELI 400mg/kg
GROUPS


ALT
250
200
150
100
50
0
mg/dl
Control
Negative Control
Standard Drug Treated
EELI 200mg/kg
EELI 400mg/kg
GROUPS

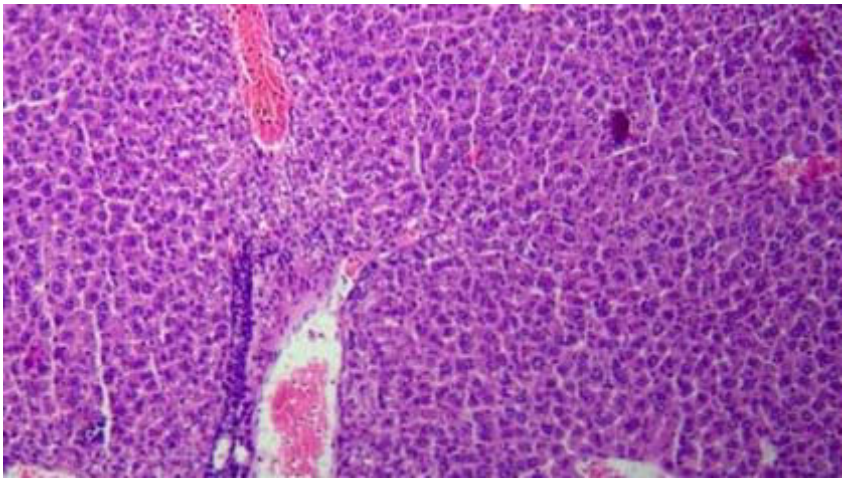

AST
250
200
150
100
50
0
mg/dl
Control
Negative Control
Standard Drug Treated
EELI 200mg/kg
EELI 400mg/kg
GROUPS


Bilurubin
2.5
2.0
1.5
1.0
0.5
0.0
mg/dl
Control
Negative Control
Standard Drug Treated
EELI 200mg/kg
EELI 400mg/kg
GROUPS


Total Protein
10
8
6
4
2
0
mg/dl
Control
Negative Control
Standard Drug Treated
EELI 200mg/kg
EELI 400mg/kg
GROUPS

HISTOPATHOLOGICAL REPORT

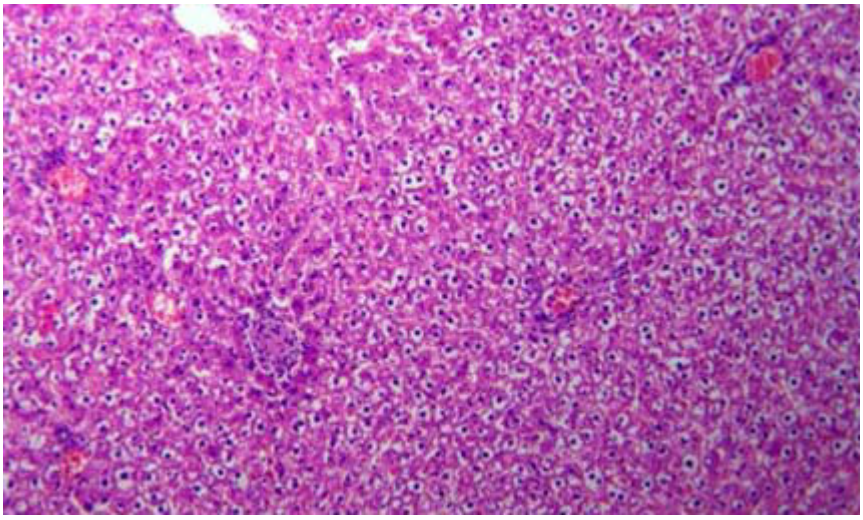
Figure: 1 Control group (Normal saline 5ml/kg)



IMPRESSION:

Section of liver in normal control group (A) shows liver parenchyma with normal architecture of hepatic cells.

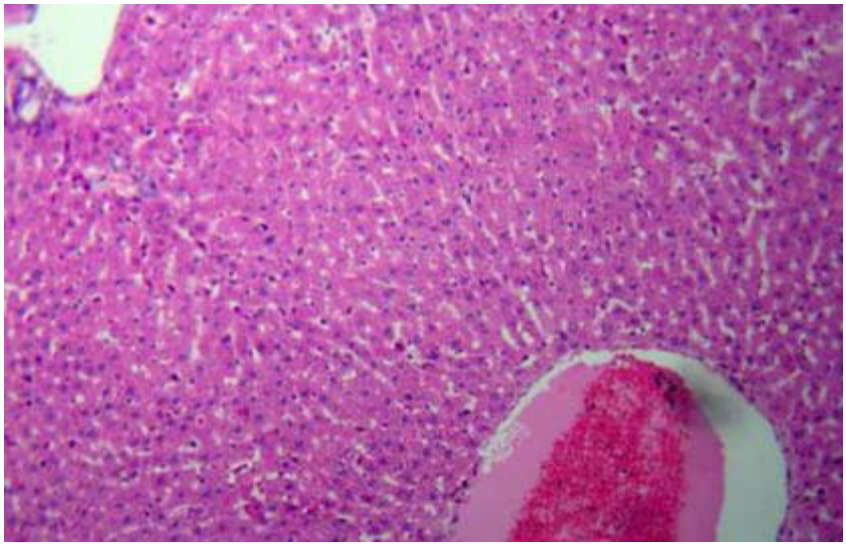
Figure: 2 Negative Control group (PCM 2gm/kg)



IMPRESSION:

Section of liver in Negative control group (B) shows partially effaced architecture. Some of the hepatocytes show apoptotic changes, perivenular mononuclear inflammatory infiltration, and scattered inflammatory infiltration with in the parenchyma.

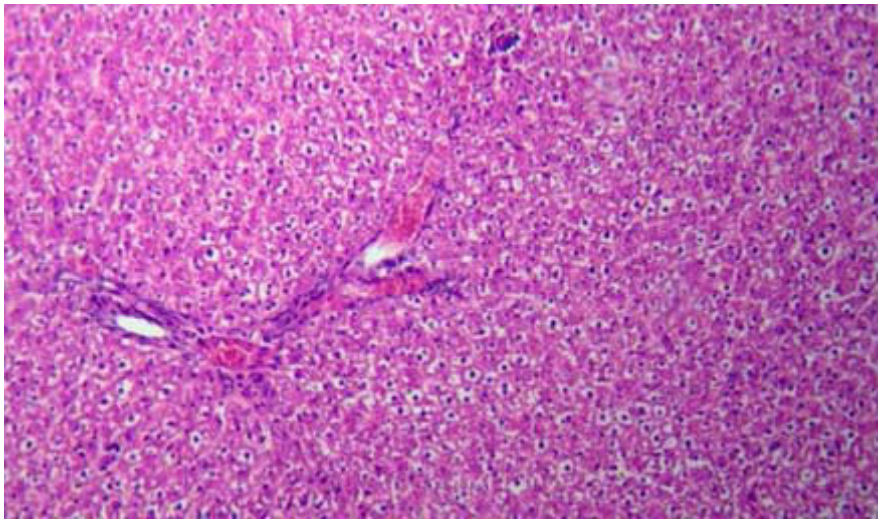
Figure: 3 Standard drug treated group (silymarin 100mg/kg)



IMPRESSION:

Section of liver in standard drug treated group (C) shows liver parenchyma with intact architecture of hepatocytes.. Some of the central veins show congestion with diffuse congestion of sinusoids.

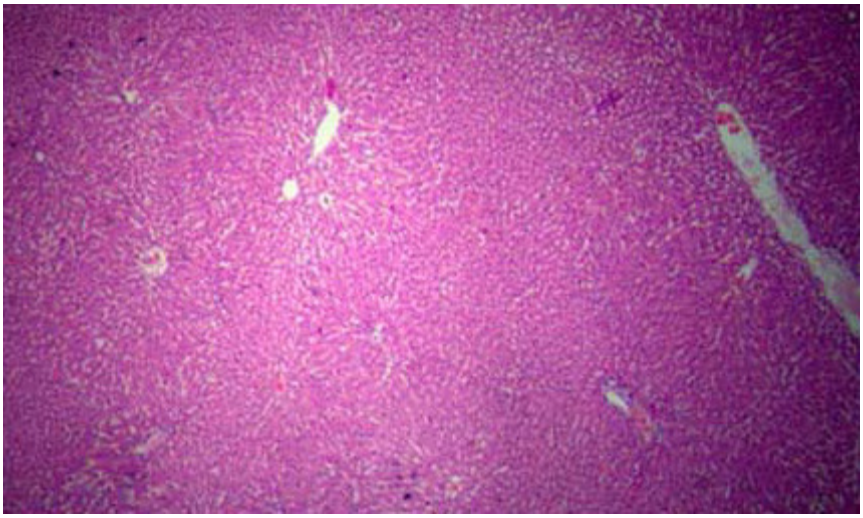
Figure: 4 Plant extract treated group (EELI 200 mg/kg)



IMPRESSION:

Plants extract treated group (D) showing mild degeneration of and reverting to regeneration of hepatocytes, sinusoidal congestion and scattered mononuclear inflammatory cells.

Figure: 5 plant extract treated group (EELI 400 mg/kg)



IMPRESSION:

Plant extract treated group (E) shows intact architecture, complete regeneration of heaptocytes, sinusidal congesion which is similar to silymarin treated(C) group.

DISCUSSIONS

The purpose of the study was exploring the hepatoprotective effect of ethanolic extract of *lawsonia inermis* leaves against paracetamol induced hepatotoxicity in rats.

The hepatotoxic effect of the paracetamol is due to formation of toxic metabolite (N-acetyl-p-benzoquinoneimine) when it is metabolised by cytochrome P-450, which causes oxidative stress and glutathione depletion.

The animals treated with paracetamol had markedly elevated values of the biochemical parameters including SGOT, SGPT, ALP, bilirubin and the level of total protein decreased compared to normal group of animals, its indicating acute hepatocellulor damage. Pretreatment with EELI (200 & 400mg/kg) made the serum values such as SGOT, SGPT, ALP significantly lower than toxic control values. The total bilirubin value was found to be significantly reversal. Which is near to the normal control value? The total protein level was significantly increased compared to the toxic control group.

From the results EELI (200 & 400mg/kg) showed significant hepatoprotective activity, as evidence by the biochemical and histopathalogical parameters. The protective effect of EELI against paracetamol induced hepatotoxicity induced rats. This effect presence of flavanoids (**Apigenin-7-glycosides)** demonstrating hepatoprotective activity in ethanolic extract of *Lawsonia inermis L.* leaves.

CONCLUSION

The hepatoprotective effect of ethanolic extract of *lawsonia inermis* leaves was confirmed by the biochemical and histopathalogical studies.

In biochemical studies serum marker enzymes such as SGOT, SGPT, ALP, bilirubin showed marked increase and decrease in the level of protein. The same is observed in liver disease in clinical practice and hence are having diagnostic importance in the assessment of liver function test.

In the present study, the ethanolic extract of *lawsonia inermis* leaves significantly reduced the elevated levels of above mensioned serum marker enzymes and increase in the level of protein. Hence at this point concluded the ethanolic extract of *lawsonia inermis* leaves posses hepatoprotective activity.

In support to this study, histopathalogical results also show significant activity of the plant. In toxicant treated animals there will be severe disturbances in the cytoarchitecture of the liver. But in ethanolic extract of *lawsonia inermis* leaves treated group animals exhibited minimal hepatic dearrangrments and intact cytoarchitecture of the liver was maintained. In addition to this there is regeneration of hepatocytes also observed, which indicating hepatoprotective activity.

Finally based on the improvement in serum marker enzyme level, and histopathalogical studies. It is concluded that the ethanolic extract of *lawsonia inermis* leaves posses hepatoprotective activity.

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सं. भा.व.स./द.क्षे.के./No. BSI/SRC/5/23/2013-14/Tech. /1796 दिनांक/Date: : 07th February 2014

सेवा में / To

Mr. N. Arivazhagan
II Year M. Pharmacy
Department of Pharmacology
Cherran college of Pharmacy
Coimbatore
Tamil Nadu

महोदय/Sir,

The plant specimen brought by you for identification is identified as *Lawsonia inermis* L. – LYTHRACEAE.

धन्यवाद/Thanking you,

भवदीय/Yours faithfully,

(डा. एम पलनिसामी /Dr. M. Palanisamy)
वैज्ञानिक सी, प्रभारी / Scientist 'C' In-charge

वैज्ञानिक 'सी' / SCIENTIST 'C'
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