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Callicarpa macrophylla Leaves Extracts Evaluated as Hepatoprotective in Wistar Rats

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ABSTRACT

Liver is one of the most important organ, which plays a provital role in regulating various physiological processes in the body. It has great capacity to detoxicate toxic substances and synthesize useful principles. *Callicarpa macrophylla* has witnessed pivotal role in herbal medicine since several years. The ethanolic & aqueous extracts of *Callicarpa macrophylla* leaves was tested for its phytochemical constituents and hepatoprotective activity against CCl_4 induced hepatotoxicity in wistar rats. The results revealed that the oral administration of CCl_4 (2 mL/kg) to the animals caused statistically significant ($p<0.05$) increases in the serum activities of SGOT, SGPT, ALP and bilirubin when compared with control group where there was decreased in level of total protein and albumin. The extract at a dose of200 & 400 mg/kg(for both extract) was able to protect against CCl_4 induced hepatotoxicity in albino rat as revealed by significant decreases in the serum level of SGOT, SGPT, ALP and bilirubin and whereas an increase in total protein concentration when compared to the CCl_4+distilled water treated group. The ethanolic extract at 400mg/kg, p.o. was found most effective among all the extract treatments when compared to CCl_4+distilled water treated groups. The further studies are needed to evaluate potential usefulness of ethanolic and aqueous extract in clinical condition associated with liver damage.

Keywords: *Callicarpa macrophylla*, CCl_4, Hepatotoxicity, Liver enzymes and bilirubin

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INTRODUCTION

The liver is the largest gland of the body playing an astonishing array of vital functions in the continuation, performance and adaptable homeostasis of the body. It is concerned with approximately all the biochemical pathway to enlargement, fight in opposition to disease, nutrient contribute, energy stipulation and reproduction [1-2]. Liver disease is one of the major causes of morbidity and mortality in public which affects humans of all ages. Due to liver disease about 20,000 deaths occur every year [3-5]. Some of the commonly known disorders of liver are viral hepatitis, alcohol liver disease, non-alcoholic fatty liver disease, autoimmune liver disease, metabolic liver disease, drug induced liver injury, gallstones etc.

Hepatocellular carcinoma is one of the ten most common tumour in the world with over 2, 50,000 new cases each year [3]. Cell damage is caused by free radicals through mechanisms of covalent binding and lipid peroxidation with subsequent tissue injury. Some synthetic compounds are currently available as hepatoprotective agents. However, such compounds may exert several side effect and disadvantages. In view of severe adverse side effects of synthetic agents, there is growing focus to develop more valuable and protected drugs which may raise the therapeutic benefits for patients [3-5]. Numerous medicinal plants and their formulations are used for liver disorders in ethno medical practices as well as in traditional systems of medicine in India [7].

Liver is involved in several vital functions, such as metabolism, secretion and storage and it is the most important organ, which plays a provital role in regulating various physiological processes in the body. It has great capacity to detoxicate toxic substances and synthesize useful principles. Liver functions as a center of metabolism of nutrients such as carbohydrates, proteins and lipids and excretion of waste metabolites. Additionally, it also handles the metabolism and excretion of drugs and other xenobiotics from the body thereby providing protection against foreign substances by detoxifying and eliminating them [9-10]. Liver cells possess the antioxidant defence system consisting of

antioxidants such as growth stimulating harmone (GSH), ascorbic acid, vitamin E and antioxidant enzymes such as superoxide dismutase (SOD), catalase and GPx to protect own cells against oxidative stress, which causes destruction of cell components and cell death. The liver is a major target organ for toxicity of xenobiotics and drugs, because most of the orally ingested chemicals and drugs first go to liver where they are metabolized into toxic intermediates.

A large number of xenobiotics are reported to be potentially hepatotoxic, Hepatocytes, which make up the majority of the liver structure, are very active in the metabolism of exogenous chemicals, and this is one of the major reasons why the liver is a target for toxic substances. During the detoxification of xenobiotics, reactive oxygen species (ROS) are generated which cause oxidative stress which leads to the hepatic damage.

Hepatotoxicity is a damage or injury to liver which is caused by various drugs, chemicals and other agents. Extent of liver damage or injury depends on degree of exposure, mild liver damage cause dysfunctioning but severe liver damage result in liver failure. The liver is largest organ in a body weighing about 1.4-1.6 kg in the males and 1.2-1.4 kg in the females. The liver receives a dual blood supply with about 20% of blood coming from the hepatic artery and 80% from the portal circulation. The blood flow to the liver is around 20 to 25% of the total cardiac output. It serves various vital functions. The most important function of liver is to filter toxic substances from the body, including alcohol and various medications like chemotherapy drugs such as antibiotics, acetaminophen etc.[11].

Liver damage is associated with cellular necrosis, increase in tissue lipid peroxidation and depletion in the tissue GSH levels. In addition serum levels of many biochemical markers like SGOT, SGPT, triglycerides, cholesterol, bilirubin, alkaline phosphatase are elevated [12].Liver disease is one of the major causes of morbidity and mortality in public, affecting humans of all ages. About 20,000 deaths occur every year due to liver disorders. Some of the commonly known disorders are viral hepatitis, alcohol liver disease, non-alcoholic fatty liver disease, autoimmune liver disease, metabolic liver disease, drug induced liver injury, gallstones, etc. Hepatocellular carcinoma is one of the ten most common tumors in the world with over 2,50,000 new cases each year [5, 13].

Biologic factors such as hepatitis virus, bile duct obstruction, cholesterol overload, schistosomiasis etc. or chemical factors such as CCl_4 administration, alcohol intake, etc. were known to contribute to liver fibrosis. Hepatic fibrosis is major features of a wide range of chronic liver injuries including metabolic, viral, cholestatic and genetic disease. The failure of bile salt excretion in cholestasis leads to retention of hydrophobic bile salts within the hepatocytes and causes apoptosis and/or necrosis [14]. Oxidative stress has been implicated in the pathogenesis of various liver diseases including alcoholic liver disease, nonalcoholic fatty liver disease, and chronic hepatitis. In many patients, hepatitis such as non-alcoholic fatty liver disease becomes chronic and eventually progresses to more serious liver pathologies, such as fibrosis, cirrhosis, or even carcinogenesis, causing devastating economic losses and mortality [15]. Drug-induced liver disease can be predictable (high incidence and dose-related) or unpredictable (low incidence and may or may not be dose-related). Unpredictable reactions, also referred to as idiosyncratic, can be viewed as either immune-mediated hypersensitivity or nonimmune reactions. Most potent predictable hepatotoxins are recognized in the animal testing or clinical phase of drug development.

Hepatotoxicity and Its Mechanism

Liver plays a central role in transforming and clearing chemicals and is consequently susceptible to the toxicity induced from these agents. Chemicals that cause liver injury are termed as hepatotoxins, and 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. Chemicals often cause subclinical injury to liver which may be manifest by abnormal liver enzyme tests. Certain medicinal agents when taken in overdoses and sometimes even when introduced within therapeutic ranges may injure the organ. Other chemical agents such as those used in laboratories and industries, natural chemicals (e.g. Microcystins) and herbal remedies can also induce hepatotoxicity (e.g. Lycopodium serratum and Ephedra equisetina).

The unique property of liver to metabolize substances and its close relationship with the gastrointestinal tract, it is highly susceptible to injury from drugs and other substances. Approximately 75% of blood reaching the liver arrives directly from gastrointestinal organs and then spleen through portal veins which bring drugs and xenobiotics in concentrated form. Numerous mechanisms may be cited to be responsible for either inducing hepatic injury or worsening the damage process. Although exact mechanism of hepatic injury remains largely unknown, it appears to involve 2 pathways that is direct hepatotoxicity and adverse immune reactions.

In most instances, hepatic injury is initiated by the bioactivation of drugs to chemically reactive metabolites, which have the ability to interact with cellular macromolecules such as proteins, lipids, and nucleic acids, leading to protein dysfunction, lipid peroxidation, DNA damage, and oxidative stress [16]. Additionally, these reactive metabolites may induce disruption of ionic gradients and intracellular calcium stores, resulting in mitochondrial dysfunction and loss of energy production. Its dysfunction releases excessive amount of oxidants which in turn injures 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 liver. This promotes further liver damage. This impairment of cellular function can culminate in cell death [17].

Hepatic cellular dysfunction and death also have the ability to initiate immunological reactions, including both innate and adaptive immune responses. Stress and damage to hepatocytes result in the release of signals that stimulate activation of other cells, particularly those of the innate immune system, including Kupffer cells (KC), natural killer (NK) cells. These cells contribute to the progression of liver injury by producing proinflammatory mediators and secreting chemokines to further recruit inflammatory cells to the liver. It has been demonstrated that various inflammatory cytokines, such as tumor Necrosis factor (TNF)-α, Interferon (IFN)-γ, and Interleukin (IL)-1β, produced during hepatic injury are involved in promoting tissue damage [18]. However, innate immune cells are also the main source of IL-10, IL-6, and certain prostaglandins, all of which have been shown to play a hepatoprotective role [19]. Thus, it is the delicate balance of inflammatory and hepatoprotective mediators produced after activation of the innate immune system that determines an individual's susceptibility and adaptation to hepatic injury.

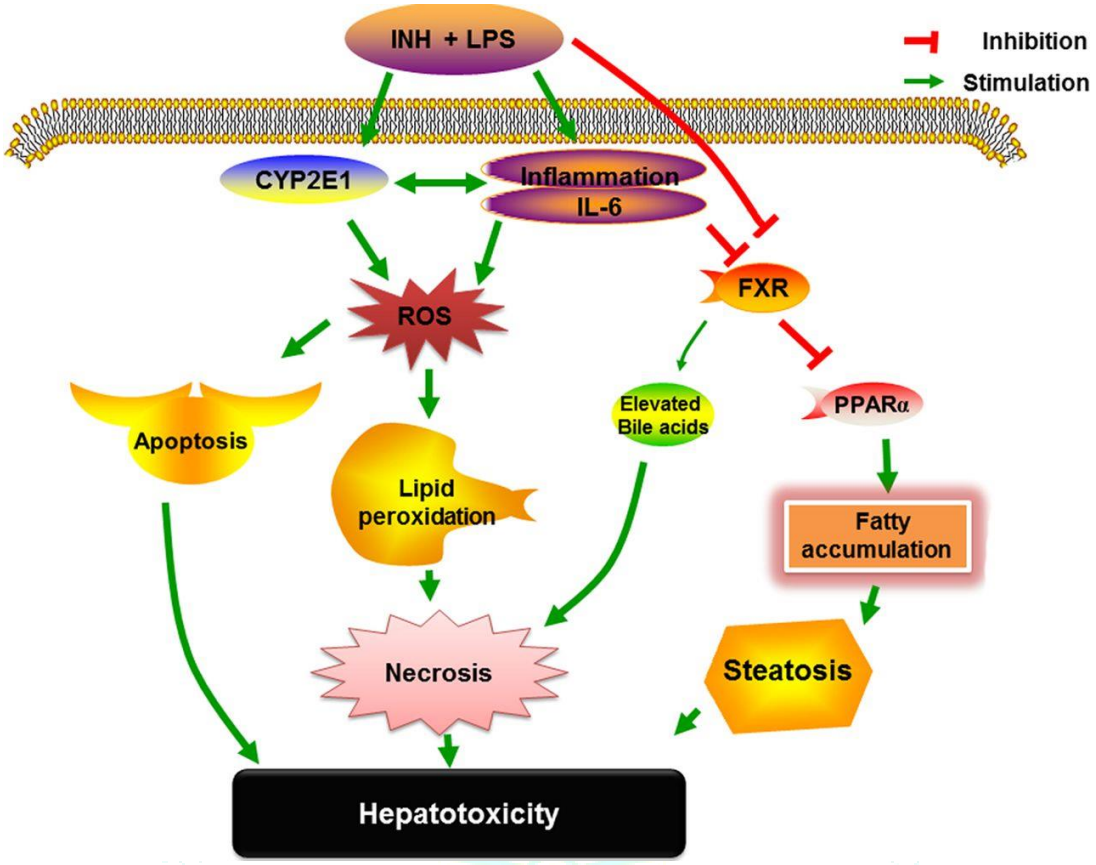

Figure 1: Mechanism of hepatic injury

Hepatoprotective Study

Natural products and their active principles as sources for new drug discovery and treatment of diseases have attracted attention in recent years. Herbs are generally considered safe and proved to be effective against various human ailments. Herbal based therapeutics for liver disorders has been used in India for a long time and has been popularized in world over by leading pharmaceuticals [20]. The currently observed rapid increase in consumption of herbal remedies worldwide has been stimulated by several factors, including the notion that all herbal products are safe and effective. The 21st century has seen a paradigm shift towards therapeutic evaluation of herbal products in liver disease models by carefully synergizing the strength of the traditional systems of medicine with that of the modern concept of evidence based medicinal evaluation, standardization and randomized placebo controlled clinical trials to support clinical efficacy [21].

Herbal drugs are more widely used than allopathic; drugs as hepatoprotective because of them are inexpensive, better cultural acceptability, better compatibility, with the human body and minimal side effects. These herbal drugs have shown the ability to maintain the normal functional statues of the liver with or without fewer side effects. The liver plays an astonishing array of vital functions in the maintenance, performance and regulating homeostasis of the body. It is involved with almost all the biochemical pathways to growth, fight against disease, nutrient supply, energy provision and reproduction. Therefore, maintenance of a healthy liver is essential for the overall well-being of an individual. Liver cell injury caused by various toxicants such as certain chemotherapeutic agents, carbon tetrachloride, thioacetamide etc. chronic alcohol consumption and microbes is well-studied. Among the many diseases that can affect the liver the most common is 'viral hepatitis' (Inflammation of liver caused by viral infection). Hepatitis can be caused by drugs, viruses, bacteria, mushrooms, parasites like amoebas or giardiasis.

Herbal drugs have gained importance and popularity in recent years because of their safety, efficacy and cost effectiveness. Several Indian medicinal plants have been extensively used in the Indian traditional system of medicine for the management of liver disorder. The use of natural remedies for the treatment of liver diseases has a long history and medicinal plants and their derivatives are still used all over the world in one form or the other for this purpose. Scientific evaluation of plants has often shown that active principles in these are responsible for therapeutic success [22]. A large number of medicinal plants have been tested and found to contain active principles with curative properties against a variety of diseases. Liver protective plants contain a variety of chemical constituents like phenols, Coumarins, Lignans, essential oil,monoterpenes, carotinoids, glycosides, flavonoids, organic acids, lipids, alkaloids and xanthenes.

Therefore a large number of plants and formulations have been claimed to have hepatoprotective activity so the development of plant based hepatic protective drugs has been given importance in the global market. This review article has been presented to enumerate some plants that have hepatoprotective properties such as *Abelmoschusmoschatus, Ageratum conyzoide, Ardisiasolanacea, Arisaema leschenaultia, Callicarpa macrophylla, Capparisspinosa,Cassia auriculata, Chenopodium album, Chrysophyllumalbidum*[23].

MATERIALS AND METHODS

To evaluate the hepatoprotective effect of *Callicarpa macrophylla* leavesextract against carbon tetrachloride induced hepatotoxicity in experimental rats, following

chemicals, reagents, diagnostic kits, equipments& instruments were used. All chemicals and reagents used were of analytical grade.

Table 1: Chemicals, Reagents & Diagnostic kits.

Chemical Name	Company Name
Carbon Tetrachloride	GalaxoSmithkline Pharmaceutical Ltd.
Liquid Paraffin	RFCL Limited
Ethanol	SD -Fine Chemicals Limited
Di-ethyl Ether	SD- Fine Chemicals Limited
Glycerine	RFCL Limited
Petroleum ether	RFCL Limited
Silymarin	Asklepios Remedies (Pvt.) Ltd.
Formaldehyde	RFCL Limited
SGOT Diagnostic Kit	Span Diagnostics Ltd.
SGPT Diagnostic Kit	Span Diagnostics Ltd.
ALP Diagnostic Kit	Span Diagnostics Ltd.
Bilirubin Diagnostic Kit	Span Diagnostics Ltd.
Albumin Diagnostic Kits	Span Diagnostics Ltd.

Extraction of Leaves

Ethanolic Extract

Coarsely powdered leaves (350gm) were extracted with Soxhlet apparatus using petroleum ether for about 12 hrs. After defatting the marc was air dried and packed into Soxhlet apparatus and further extracted with 95% ethanol. The solvent were removed from the extracts under reduced pressure by using rotator vacuum evaporator. The percentage yield of ethanolic extract was found to be 7.5%.

Aqueous Extract

Coarsely powdered leaves (350gm) was taken into a round bottom flask (200 mL) and macerated with into 500mL of distilled water and 10mL of chloroform (preservative) for 24 h with occasional shaking for every hour. Then the marc was removed by filtering the extract and the extract was concentrated on a water bath maintained at 50^{0}C. The percentage yield of ethanolic extracts was found to be 6%.

Experimental Animals

Wistar rats of (180-200 gm) of either sex were obtained from animal house for evaluation of hepatoprotective effect of *Callicarpa macrophylla* against CCl_4 induced hepatic injury in rats.Animal were kept at room temperature 23±2 ºC controlled humidity condition (50-55 %) and 12 hrs light and dark cycles. They were caged with a maximum of three animals in each polypropylene cage and were fed with standard food pellets and water.

Acute Toxicity

Acute toxicity was performed as per OECD guideline No.420. Female rats were dosed in a stepwise procedure using the fixed doses of aqueous and ethanolic extracts of *Callicarpa macrophylla* leaves5, 50, 300 and 2000 mg/kg and after dosing animals were observed for signs and conditions associated with pain, suffering, behavior and mortalityat first 30 minutes and every 4 hours periodically during the first 24 hours and daily thereafter for a total of 14 days.

Induction of Hepatotoxicity

Carbon tetrachloride (CCl_4) was used for inducing hepatotoxicity. Liver damage was induced in rat with 1:1 (v/v) mixture of CCl_4 in liquid paraffin, administered intra-peritoneal at a dose of 2 mL/ kg on 9th day of the study.

Experimental Design for Hepatoprotective Activity

Animals were randomly divided into seven groups of six animals each and treated orally, once daily for nine days in the following manner.Group I-served as normal control and received distilled water (5mL/kg).Group II-served as toxic control and received distilled water (5mL/kg) and 1:1 (v/v) mixture of CCl_4 in liquid paraffin (2 mL/kg, i.p) on 9th day. Group III-served as standard group and received Silymarin (50 mg/kg) and 1:1 (v/v) mixture of CCl_4 in liquid paraffin (2 mL/kg, i.p) on 9th Day. Group IV was treated with ethanolic extract of *Callicarpa macrophylla* leaves (200 mg/kg/Day) and 1:1 (v/v) mixture of CCl_4 in liquid paraffin (2 mL/kg, i.p) on 9th Day. Group V was treated with ethanolic extract of *Callicarpa macrophylla* leaves *(*400 mg/kg/Day) and 1:1 (v/v) mixture of CCl_4 in liquid paraffin (2 mL/kg, i.p) on 9th day.Group VI was treated with aqueous extract of *Callicarpa macrophylla* leaves (200 mg/kg, Day) and 1:1 (v/v) mixture of CCl_4 in liquid paraffin (2mL/kg, i.p) on 9th Day. Group VII was treated with aqueous extract of *Callicarpa macrophylla* leaves (400 mg/kg, Day) and 1:1 (v/v) mixture of CCl_4 in liquid paraffin (2 mL/kg, i.p) on 9th Day.

Estimation of Liver Function Enzymes

To determine the functional state of liver, after 24 hours of CCl_4 administration (10th Day), blood samples were collected by retro-orbital plexus puncture under mild ether anesthesia. The collected blood was allowed to clot at room temperature and serum was separated by centrifugation at 2500 rpm for 15 min. Then serum was used for the estimation of biochemical parameters such as serum glutamate oxaloacetate transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), alkaline phosphatase (ALP), serum total bilirubin (TB) and total protein (TP).The biochemical parameters were estimated as per the standard procedure prescribed by manufacturer's instruction manual provided in the standard kit using autoanalyser.

Estimation of Serum Glutamate Oxaloacetate Transaminase (SGOT)

Method: 2, 4-DNPH (Reitman and Frankel Method)

Principle:Aspartate aminotransferase (AST) catalysis the transamination of L- Aspartate and α- Ketoglutamate (α-KG) to form Oxaloacetate and L- Glutamate. Oxaloacetate so formed is coupled with 2, 4- Dinitrophenyl hydrazine (2, 4-DNPH) to form a corresponding hydrazone, a brown colored complex in alkaline medium and this can be measured calorimetrically.

α- KG + L- Aspartate ⟷ Oxaloacetate + L- Glutamate

Oxaloacetate+X2, 4- DNPH ⟷ Corresponding Hydrazone

Reitman and Frankel Method is an end-point colorimetric method for the estimation of enzymes activity. To obtain accurate results, method has been standardized with Kinetic Method (Standard Karmen unit assay). This product is single point calibration version of original method for maximum ease of use and convenient.

Reagents:

S. No.	Reagents
1.	Buffered Aspartate- α-KG Substrate, pH 7.4
2.	2,4-DNPH Colour Reagent
3.	Sodium Hydroxide, 4N
4.	Working Pyruvate Standard, 6mM (114 IU/L)

Preparation of Working Reagent:

Reagent 1, 2 and 4 were ready to use andsolution 1: Dilute 1mL of Reagent 3 to 10 mL with purified water.

Procedure

Pipette into tube marked	Blank	Standard	Test	Control
Reagent 1	0.25mL	0.25mL	0.25mL	0.25mL
Serum	-	-	0.05mL	-
Standard	-	0.05mL	-	
Mix well and incubate at 37^{0}C for 60 minutes				
Reagent	0.25mL	0.25mL	0.25mL	0.25mL
Deionised water	0.05mL	-	-	-
Serum	-	-	-	0.05mL
Mix well and allow to stand at Room Temperature (150^0 -30 ^{0}C) for 20 minutes				
Solution 1	2.5mL	2.5mL	2.5mL	0.05mL

Mix well and read the O.D (Optical Density) against purified water in a Colorimeter using green filter or on Photometer at 505 nm, within 15 minutes.

Calculation:

$$\text{AST (GPT) activity} = \frac{O.D.\ of\ \ Test - O.D.of\ control}{O.D.of\ Standard - O.D.of\ Blank} \times Conc.\ of\ Standard$$

Estimation of Serum Glutamate Pyruvate Transaminase (SGPT)

Method: 2, 4-DNPH (Reitman and Frankel Method)

Principle: Alanine aminotransferase (ALT) catalysis the transamination of L-Alanine and α- Ketoglutarate (α-KG) to form Pyruvate and L- Glutamate. Pyruvate so formed is coupled with 2, 4- Dinitrophenyl hydrazine (2, 4-DNPH) to form a corresponding hydrazone, a brown colored complex in alkaline medium and this can be measured calorimetrically.

α- KG + L- Alanine ⟶ Pyruvate + L- Glutamate

Pyruvate + 2, 4- DNPH ⟷ Corresponding Hydrazone (Brown color)

Reitman and Frankel Method is an end-point colorimetric method for the estimation of enzymes activity. To obtain accurate results, method has been standardized with Kinetic Method (Standard Karmen Unit Assay). This product is single point calibration version of original method for maximum ease of use and convenience.

Reagents:

S. No.	Reagents
1.	Buffered Aspartate- α-KG Substrate, pH 7.4
2.	2,4-DNPH Colour Reagent
3.	Sodium Hydroxide, 4N
4.	Working Pyruvate Standard, 8mM (150 IU/L)

Preparation of Working Reagent:

Reagent 1, 2 and 4 were ready to use and solution 1: Dilute 1mL of Reagent 3 to 10 mL with purified water.

Procedure:

Pipette into tube marked	Blank	Standard	Test	Control
Reagent 1	0.25mL	0.25mL	0.25mL	0.25mL
Serum	-	-	0.05mL	-
Standard	-	0.05mL	-	
Mix well and incubate at 37^{0}C for 60 minutes				
Reagent	0.25mL	0.25mL	0.25mL	0.25mL
Deionised water	0.05mL	-	-	-
Serum	-	-	-	0.05mL
Mix well and allow to stand at Room Temperature (150^0 -30 ^{0}C) for 20 minutes				
Solution 1	2.5mL	2.5mL	2.5mL	0.05mL

Mix well and read the O.D (Optical Density) against purified water in a Colorimeter using green filter or on Photometer at 505 nm, within 15 minutes.

Calculation:

$$\text{ALT (GPT) activity} = \frac{O.D.of\ Test - O.D.of\ control}{O.D.of\ Standard - O.D.of\ Blank} \times Conc\ of\ standard$$

Estimation of Serum Alkaline Phosphatase (ALP)

Method: Kind and King's

Principle: Alkaline Phosphatase from serum converts Phenyl Phosphate to Inorganic Phosphate and Phenol at pH 10.0. Phenol so formed reacts in alkaline medium with 4-Aminoantipyrine in presence of the oxidizing agent Potassium Ferricyanide and forms an Orange-Red colored complex, which can be measured colorimetrically. The color intensity is proportional to the enzyme activity.

The Reaction can be represented as:

$$\text{Phenyl Phosphate} \xrightarrow[pH\ 10]{\text{Alkaline Phosphatase}} \text{Phenol + Phosphate}$$

$$\text{Phenol + 4-Aminoantipyrine} \xrightarrow[OH]{\text{Potassium Ferricynade}} \text{Orange-Red complex } (\lambda \text{ max= 510-520 nm}).$$

Reagents:

Reagent 1: Buffered Substrate, pH 10.0

Reagent 2: Chromogen Reagent

Reagent 3: Phenol Standard, 10 mg%

Preparation of Working Reagent:

Reconstitute one vial of Reagent 1, Buffered substrate with 2.2 mL of Purified Water.

Reagents 2 and 3 were ready to use.

Procedure:

Pipette into marked	Blank (B)	Standard (S)	Control (C)	Test (T)
Working buffered	0.5mL	0.5mL	0.5mL	0.5mL
Purified water	1.5mL	1.5mL	1.5mL	1.5mL
Mix well and incubate at 37^0 C for 60 minutes				
Serum	-	-	-	0.05mL
Reagent 3	-	0.05mL	-	-
Mix well and allow to stand at Room Temperature(15-30^{0}C) for 20 minutes				
Reagent 2	1.0mL	1.0mL	1.0mL	1.0mL
Serum	-	-	0.05mL	-

Mix well after addition of each Reagent and measure the O.D (Optical Density) of Blank (B), Standard (S), Control (C) and Test (T) against Purified Water using a Green filter.

Calculation:

$$\text{Serum Alkaline Phosphate activity} = \frac{O.D.Test - O.D.Control}{O.D.Control - O.D.Blank} \times 10$$

Estimation of Serum Bilirubin (BIL)

Method: Jendrassik and Grof Method.

It was estimated by using commercially available standard kit supplied by Span Diagnostics Ltd. Surat (India).

Principle: Both conjugated and unconjugated bilirubin reacts with diazotized sulphanilic acid. Reaction with unconjugated bilirubin is hastened by the use of caffeine sodium benzoate as accelerator for Serum Bilirubin estimation. The pink colour developed was measured at 540 nm and the intensity of colour formed is directly proportional to bilirubin concentration in the sample.

Reagents:

Reagent 1: Nitrite Reagent; Sodium Nitrite 10mM

Reagent 2: Sulphanilic acid; Suphanilic acid 26 mM and Hydrochloric acid 180 mM

Reagent 3: Accelerator; Sodium Benzoate 340 mM and Caffeine 200 mM

Assay Parameters

Reaction Type - End Point, Reaction Time - 5 min. at R.T. , Wavelength -540 nm, Zero Setting with-Serum Blanks, Sample volume-0 50 µL, Reagent volume-1.02 mL, Factor-20.2, Linearity-20 mg/dl

Procedure:

	Direct		Total	
Pipette into tube marked Reagent	Sample blank (B_1)	Test (T_1)	Sample blank (B_2)	Test (T_2)
Reagent 1	-	50	-	50 mL
Reagent 2	0.1mL	0.1mL	0.1mL	0.1mL
Sample	0.1mL	0.1 mL	0.1mL	0.1mL
Reagent 3	-	-	1.0mL	1.0 mL
Saline	1.0mL	1.0mL	-	-

Mix and keep the assay mixture for 5 minutes of direct bilirubin and 10 minutes for Serum Bilirubin Read before 8 minutes the absorbance of the test against their respective blanks at 540 nm (530 – 570 nm).

Calculation:

Bilirubin (Total or Direct)

Mg (%) = (Abs Test - Abs Blank) × 20.2

Estimation of Serum Total Protein (TP) & Albumin (ALB)

Method using (Span diagnostics kit)

Principle: Protein reacted with cupric ions in alkaline medium to from a violet colored complex. The intensity of the complex was measured at 530 nm against reagent blank 0.01 mL of the standard solution, which was treated in the same way.

Procedure for total protein:

The reagents used were from span diagnostic kit.1 mL of working reagent was mixed with 0.01 mL of serum and absorbed at 530 nm. The reagent blank 0.01 mL of standard solution was treated in same way. Mix well after the addition of each reagents estimated with help of chemoanalyser and expressed as mg/ dl.

Procedure for albumin:

The reagents used were from span diagnostics kit and absorbed at 630nm. The reagent blank, 0.01 mL of standard solution was treated in same way. Mix well after the addition of each reagent Estimated with the help of chemoanalyser and expressed as mg/dl.

Statistical Analysis:

All the data are expressed as Mean ± S.E.M. One way analysis of variance (ANOVA) was used for the statistical analysis of data. Dunnett's t comparison test was used for determining the significance. A probability value of $p<0.05$ was considered as significant.

RESULT AND DISCUSSION

Result

Selection of Doses

The doses were selected on the basis of acute toxicity study which is about 1/10 & 1/5 of maximum tolerated safe dose 2000mg/kg i.e. 200 & 400 mg/kg for both extracts respectively.

Hepatoprotective Activity of Extracts

The hepatoprotective potential of ethanolic and aqueous extract of *Callicarpa macrophylla leaves*

was evaluated against carbon tetrachloride (CCl4) induced acute hepatic injury in Wistar rats.Briefly, for the purpose, experimental rats of Group-I & II were administered with distilled water (5 mL/kg) and Group II served as a toxic control and treated with 1:1 (v/v) mixture of CCl_4 in liquid paraffin (2 mL/kg, i.p) on 9th day whereas rats of Group-III received standard drug (Silymarin, 50 mg/kg), animals of test Group-IV & V received aqueous extract of *Callicarpa macrophylla* leaves at dose of 200 & 400 mg/kg and animal of test Group VI & VII received ethanolic extract of *Callicarpa macrophylla* leaves at dose of 200 & 400 mg/kg respectively. All treatments were given once daily by oral route, for nine days. Further, on ninth day of the study all rats of all groups except normal control group were administered with single intraperitoneal dose of CCl_4 (2 mL/kg) dissolved in liquid paraffin (1:1). Then on tenth day of the study (after 24 hours of CCl_4 administration) blood samples were collected and analyzed for hepatic function biomarkers in serum.

Carbon tetrachloride is well known toxic substance which is readily used to induce hepatic damage on experimental basis. As exhibited from Table 8,in carbon tetrachloride toxic control group, the SGOT, SGPT, ALP and serum bilirubin level, increase significantly, when compared to the normal control group confirming carbon tetrachloride liver toxicity. The oral pretreatment for 9 days with aqueous and ethanolic extract of *Callicarpa macrophylla* at dose of 200 & 400 mg/kg results in significantly decrease ($p<0.001$, $p < 0.01$) in the level of SGOT, SGPT in the aqueous and ethanol extract of *Callicarpa macrophylla* leaves against carbon tetrachloride control group.

A significant reduction in the level of ALP ($p<0.001$, $p< 0.01$) was observed in the group treated with ethanolic and aqueous extract of *Callicarpa macrophylla* leaves against carbon tetrachloride control group.The serum bilirubin level was decreased by aqueous and ethanol extract of *Callicarpa macrophylla* leaves shows significance ($p <0.001$, $p < 0.01$) against carbon tetrachloride control group.

Also, there was statistically significant decrease in albumin and total protein in serum of experimental rats of $CCl_4$and vehicle treated group whereas the animals of groups pretreated with Silymarin (50 mg/kg), ethanolic and aqueous extract (200 & 400 mg/kg, for both extract) when compared to that of normal control showed less reduction in serum albumin and total protein which is an indicative of hepatoprotective effect of *Callicarpa macrophylla* leaves extract.

Table 2: Effect of Ethanolic and Aqueous extract of *Callicarpa macrophylla* leaves on liver function biomarkers in CCl_4 induced hepatic injury in rats.

Parameter	SGOT (IU/L)	SGPT (IU/L)	ALP (IU/L)	Serum Bilirubin (mg/dl)	Serum Albumin (mg/dl)	Total Protein (mg/dl)
Control (5 mL/kg)	64.31±2.67	51.25±4.63	173.53±1.87	0.41±0.03	6.37±0.45	7.25±0.12
CCl_4(2 mL/kg)	149.17±.4.35	127.35.± 5.21	347.63±7.27	1.71±0.04	4.15±0.31	3.51±0.09
Silymarin (50mg/kg)	70.35±3.41***	51.45±3.61***	181.93±2.31***	0.51±0.02***	5.17±0.03***	7.01±0.11***
Ethanolic extract (200 mg/kg)	113.25±2.3^{NS}	105.13±3.61*	25.74±3.7*	0.85±0.01*	4.31±0.35*	5.61±0.03*
Ethanolic extract (400 mg/kg)	71.53±1.39***	51.17±1.31***	191.73±5.41***	0.49±0.03**	5.05±0.03**	5.79±0.04**
Aqueous extract (200 mg/kg)	127.35±2.61*	114.37±3.15^{NS}	299.12±1.31^{NS}	1.03±0.1^{NS}	3.91±0.07*	4.37±0.02*
Aqueous extract (400 mg/kg)	86.31±1.52**	63.67±1.23**	215.41±4.21**	0.63±0.03**	4.51±0.13**	5.49±0.13***

Values are mean ± SEM, N = 6, Statistical analysis carried by ANNOVA followed by Dunnet "t" Test. ^{NS}p> 0.05, *p< 0.05, **p< 0.01, ***p < 0.001.

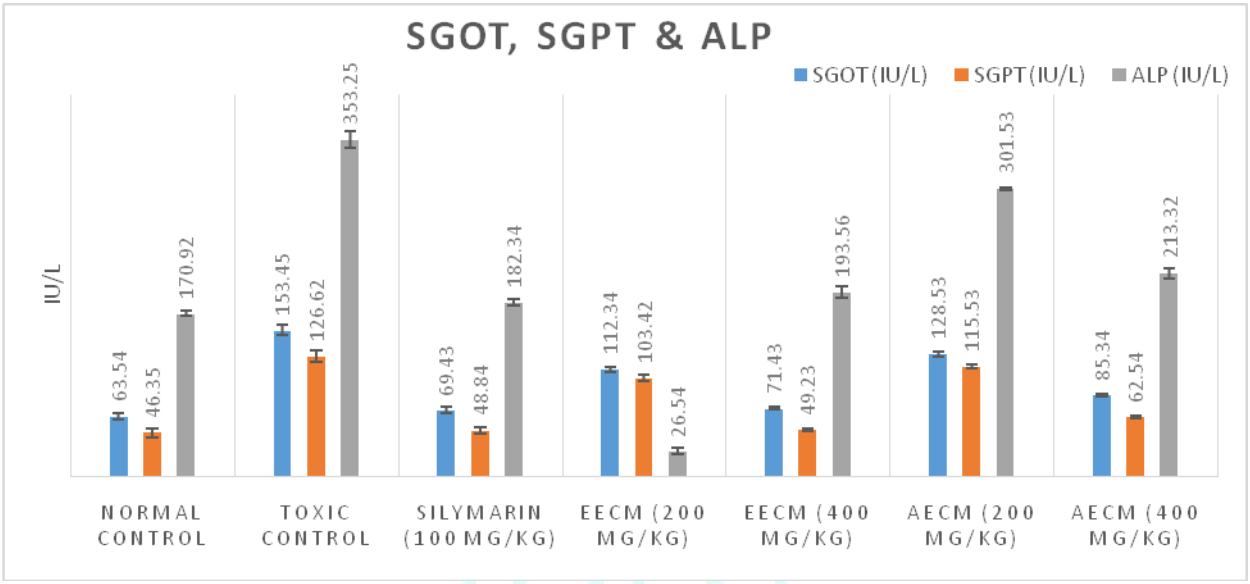

Figure 2: Effect of Silymarin, aqueous and ethanolic leaves extract of *Callicarpamacrophylla* treatment on SGOT, SGPT&ALP level in CCl_4 induced hepatic injury in rats. (Columns represent MEAN ± SEM for n= 6/group).

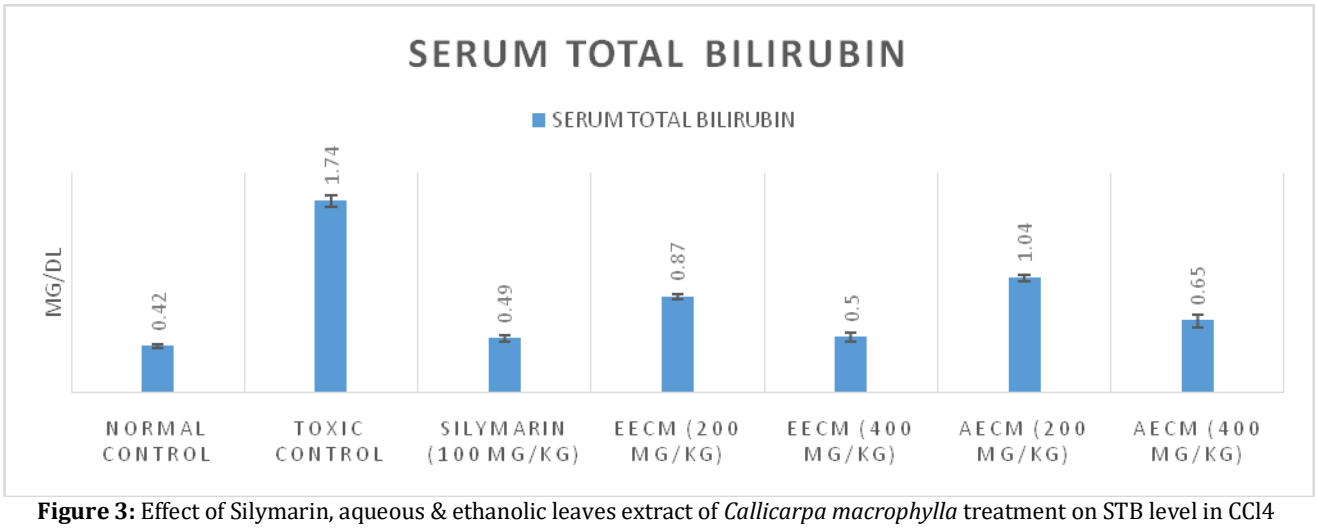

Figure 3: Effect of Silymarin, aqueous & ethanolic leaves extract of *Callicarpa macrophylla* treatment on STB level in CCl4 induced hepatic injury in rats. (Columns represent MEAN ± SEM for n= 6/group).

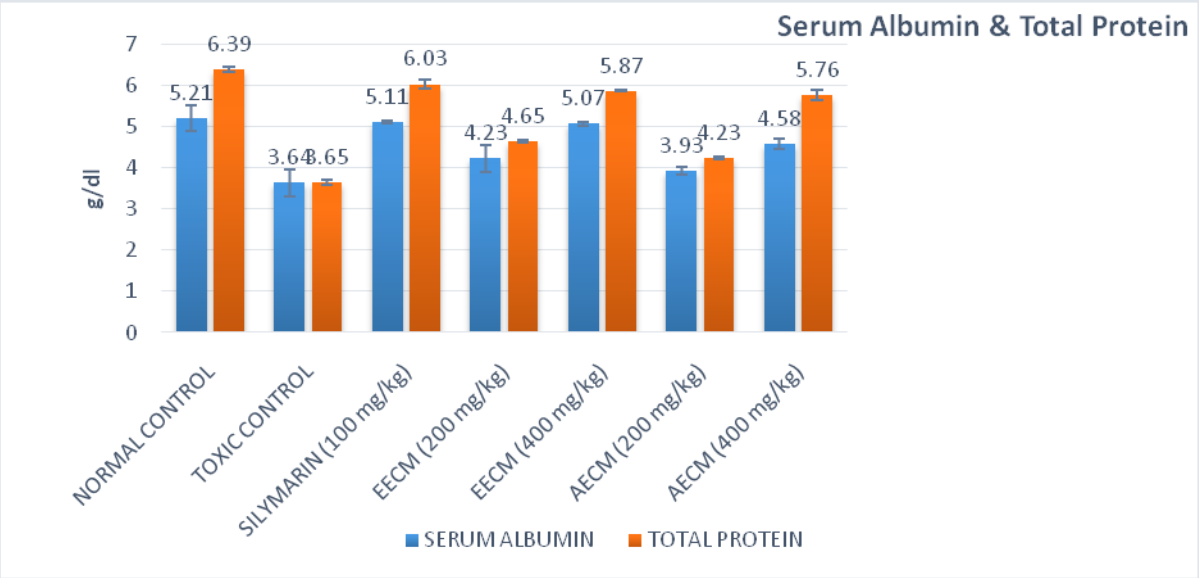

Figure 4: Effect of Silymarin, aqueous & ethanolic extract of *Callicarpa macrophylla* treatment on serum albumin & total protein level in CCl_4 hepatic injury in rats. (Columns represent MEAN ± SEM for n= 6/group).

DISCUSSION

The aqueous and ethanolic extract of the leaves *Callicarpa macrophylla* subjected for phytochemical study showed the presence of alkaloids, phenols, steroids, glycosides, phytosterols, fixed oil. The ethanolic and aqueous extract did not show any sign and symptoms of toxicity up to 2000 mg/kg dose. The effect of oral pretreatment ethanolic and aqueous extract on SGOT, SGPT, ALP, total serum bilirubin, albumin and total protein were evaluated.

CCl_4 is a well-known hepatotoxic agent and the preventive action of liver damage by CCl_4 has been widely used as an indicator of liver protective activity of drugs in general [24]. Hepatotoxicity induced by CCl_4 is the most commonly used model system for the screening of hepatoprotective activity of plant extracts/drugs [25]. The changes associated with CCl_4-induced liver damage are similar to that of acute viral hepatitis [19]. Toxicity begins with the changes in endoplasmic reticulum, which results in the loss of metabolic enzymes located in the intracellular structure. CCl_4 is a xenobiotic that produces hepatotoxicity in various experimental animals. CCl_4 is metabolized by cytochrome P450 to form a reactive trichloromethyl radical (CCl_3) and a trichloromethyl peroxyl radical (CCl_3O_2). Both radicals are capable of binding to DNA, lipids, proteins or carbohydrates, leading to lipid peroxidation, cell necrosis, and excessive deposition of collagen in liver and liver fibrosis.

The effect of CCl_4 is generally observed after 24 h of its administration. Hence the withdrawal of the blood for biochemical parameters should be carried out only after 24 h of CCl_4 intoxication. The total protein and albumin levels decreased due to the hepatotoxin intoxication. The reduction is attributed to the damage produced and localized in the endoplasmic reticulum which results in the loss of P450 leading to its functional failure with a decrease in protein synthesis and accumulation of triglycerides. In the present study, CCl_4 intoxication reduced the serum total protein and albumin levels. The pretreatment of WFM restored the total protein and albumin levels. The rise in protein and albumin level suggests the stabilization of endoplasmic reticulum leading to protein synthesis.

In this study, significant increase in SGOT, SGPT, serum bilirubin and ALP levels in the serum was observed after administration of CCl_4. The increased levels of SGOT, SGPT,serum bilirubin and ALP decreased by pretreatment with ethanolic and aqueous extracts of *Callicarpa macrophylla* leaves. Reduction in the levels of SGOT, SGPT, serum bilirubin and ALP towards the normal value is an indication of as repair of hepatic tissue damages caused by CCl_4.

The rise in marker enzymes level in CCl_4 treated animals has been attributed to damaged structural integrity of the liver. Administration of the preserved this structural integrity of the hepatocellular membrane as evidenced from attenuation of the marker enzymes level when compared to CCl_4 treated animals. It was further confirmed by the histopathological assessment of the liver tissue. Many studies have demonstrated that the hepatoprotective effect of plant extracts may be related to its antioxidant capacity to scavenge reactive oxygen species [26-28]. CCl_4 intoxication reduced the total protein level in liver homogenate, which restored significantly with the pretreatment with ethanolic and aqueous extract (200 & 400 mg/kg, p.o) The possible hepatoprotective mechanism of *Callicarpa macrophylla* leavesextract against CCl_4-induced liver injury may be through the following actions; inhibition of the cytochrome P-450 activity, prevention of the process of lipid peroxidation, stabilization of the hepatocellular membrane and enhancing the protein synthesis. The results indicate that the aqueous and ethanolic extract of *Callicarpa macrophylla leaves* has significant hepatoprotective activity. This may be probably due to the higher contents of flavonoids, flavones and flavan-3-ols.

CONCLUSION

The present study reports the hepatoprotective activity of *Callicarpa macrophylla* leaves extract on CCL_4 induced hepatotoxicity in rats. Phytochemical screening revealed the presence of glycosides, flavonoids, alkaloids, phytosterols, saponins and phenolic compounds in the extracts. Several investigators have shown that flavonoids are mainly responsible for hepatoprotective potential in various experimental animal models. Thus, it can be interpreted that the significant hepatoprotective effect may be due to

presence of flavonoids.On the basis of results obtained it can be concluded that administration (200 mg/kg and 400 mg/kg for both extract) of *Callicarpa macrophylla* leaves extract treated rats showed significant reduction of SGOT, SGPT, ALP, and bilirubin when compared with hepatotoxin treated rats, and the level of total protein and albumin were significantly increased.It can be concluded that the ethanolic extract of *Callicarpa macrophylla* leaves seems to have significant hepatoprotective activity in rats. The further studies are needed to evaluate potential usefulness of ethanolic and aqueous extract in clinical condition associated with liver damage.

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