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Research Article

Hepatoprotective effect of methanolic extracts of Phyllanthus virgatus against carbon tetrachloride-induced liver injury in rats

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ABSTRACT

In this study, the methanolic extract of Phyllanthus virgatus was evaluated against carbon tetrachloride (CCl_4) induced hepatotoxicity in rats. The results of this study indicated that Phyllanthus virgatus exhibited moderate protective effect at a dose of 100-200 mg/kg by lowering serum level of liver enzymes such as alanine amino transferees (ALT), glutamate pyruvate transaminases (SGPT), aspartate aminotransferase (AST), glutamate oxaloacetate transaminases (SGOT), and total protein to a significant extent. Further, no significant effects were seen on blood serum level at a dose of 100-200 mg/kg body weight. The highest activity was observed at a dose of 200 mg/kg with a reduction of serum concentration of ALT, AST, total bilirubin and total protein. The methanolic extract of P. virgatus showed significant decrease in the levels of liver enzymes, indicating the protection of hepatic cells thereby protecting against CCl_4 induced hepatocellular injury.

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INTRODUCTION

India is a land of diversity. Just as the diversity in its culture and heritage is the diversity in its natural resources. The most important of these resources is the forest resource. Our country is endowed with a huge forest wealth bestowed with a wide range of plants that are medicinally active. In India, there are about 45,000 plant species and among them, several thousands have been claimed to possess medicinal properties [1]. The use of medicinal plants with therapeutic properties is as old as human civilization. Various written records about medicinal plants that date back at least 5000 years support the use of mineral, plant and animal parts as source of drugs since antiquity. Texts of ancient Egyptians, Sumerians, and Greeks written 3100 BC precisely described the natural remedies in their herbal texts. Our ancestors have long back had the quest or

pursuit to explore these medicinal plants and were successful in their attempt. The diverse culture of our country is a rich source of traditional medicines, many of which are of plant origin. These include traditional system of medicines like Ayurveda, Siddha and Unani, mention the use of medicinal plants for the treatment of various human ailments. All these forms are indigenous to India and provide a large scope for scientific research.

With the advances in the field of science and technology, came the mostly used drugs of the day, the synthetic drugs. Though effective they possess side-effects and sometimes life threatening adverse effects. Hence, during the past several decades there has been a global trend for the revival of interest in the traditional system of medicine. Simultaneously, the need for basic scientific investigations of medicinal plants using indigenous medical systems has become more interesting and relevant [2].

Liver is the key organ regulating homeostasis in the body. It is involved with almost all the biochemical pathways related to growth, fight against disease, nutrient supply, energy production and reproduction. Because of its unique metabolism and relationship to the gastrointestinal tract, the liver is an important target of the toxicity of drugs, xenobiotics and oxidative stress [3]. More than 900 drugs, toxins and herbs have been reported to cause liver injury and drugs account for 20% - 40% of all instances of liver failure. In the absence of reliable liver protection drugs in modern medicine, a large number of medicinal preparations are recommended for the treatment of liver disorders and quite often claimed to offer significant relief. Attempts are being made globally to get scientific evidences for these traditionally reported herbal drugs. This scenario proves a

severe necessity to carry out research works related to hepatotoxicity.

The use of natural remedies for the treatment of liver diseases has a long history, starting with the Ayurvedic treatment, and extending to the Chinese, European and other systems of traditional medicines. The 21st century has seen a paradigm shift towards therapeutic evaluation of herbal products in liver diseases by carefully synergizing the strengths of the traditional systems of medicine with that of the modern concept of evidence-based medicinal evaluation, standardization of herbal products and randomized placebo controlled clinical trials to support clinical efficacy [4]. In spite of tremendous advances in modern medicine, no effective drugs are available that stimulate liver function and offer protection to the liver from the damage or help to regenerate hepatic cells [5].

Oxidative stress has been identified as one of the major cause of various disorders including hepatotoxicity. During recent years, it has been well known that excessive free radical generation takes place in liver injury and these observations indicate that antioxidants may be used to prevent free radicals-induced liver damages. During the past few decades, a large number of naturally occurring phenolic compounds have been identified as antioxidants, which are viewed as promising therapeutic agents for the treatment of these free radical-mediated inflammatory diseases. Several reviews have addressed the anti-inflammatory activity of phenols, attributing their property not only to the antioxidant capacity, but also to inflammatory Plants have always been an exemplary source of drugs and many of the available drugs have been derived directly or indirectly from them[6-8]. Thus, we have focused our attention on plants containing flavonoids and tannins as major chemical constituents since they have been proved of their anti-oxidant potential. In our review of literature, we found that the plant '*Phyllanthusvirgatus*' contains tannins and flavonoids as the major chemical constituents [9] and thus has the antioxidant potential. It is also well documented that carbon tetrachloride (CCl_4)triggers hepatic and renal changes in animals and man. Their mechanism of action is also very well illustrated by several authors and hence we have opted for the same to induce hepatotoxicity. Therefore, in the present study, we report the effects of methanolic extracts of Phyllanthus virgatus against CCl_4-induced hepatotoxicity in rats.

Material and Methods

Chemicals

Silymarin (Sigma), carbon tetrachloride and all other reagents used were of analytical grade. Diagnostic kits used in this study were procured from Span Diagnostics Ltd., India and Excel diagnostics Ltd., India.

Plant material

Phyllantusvirgatus whole plant was collected from Tirupati, Andhra Pradesh. The plant was identified and authenticated by Dr. K. Madhava Chetty, Assistant Professor, Department of Botany, Sri Venkateswara University, Trupati, and Andhra Pradesh. After authentication, plant was cleaned and milled into coarse powder by a mechanical grinder.

Preparation of methanolic extract of *Phyllantus virgatus* whole plant

Phyllanthusvirgatus whole plant (1kg) was extracted with 95% methanol using a soxhlet apparatus. The methanolic extract was filtered and concentrated by distillation process. A brownish green color residue was obtained (yield 6.79% w/w) and was kept in desiccator. This methanolic extract of *Phyllantus virgatus* whole plant was used for further experiments

Phytochemical screening

The extract was then subjected for the phytochemical screening [10].Thefollowingtestswerecarriedoutonthestandardizedherbalextracttodetect various phytoconstituents present in them study were procured from Span Diagnostics Ltd., India and Excel diagnostics Ltd., India

Animals

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

All experiments and protocols described in present study were approved by the Institutional Animal Ethical Committee (IAEC) of Albino Research & Training Institute, Kukatpally, Hyderabad and with permission from Committee for the purpose of Control and Supervision of Experiments on Animals (1722/RO/ERe/S/13/CPCSEA), Ministry of Social Justice and Empowerment, Government of India.

All the experiments were performed in the morning according to current guidelines for the care of laboratory animals and the ethical guidelines for the investigation of experimental pain in conscious animals.

Acute toxicity studies

Acute oral toxicity study was performed as per OECD-423 guidelines. Wistar albino rats (n = 6) of either sex selected by random sampling technique were used for acute toxicity study. The animals were kept fasting for an overnight provided with only water, after which the extract was administered orally at the dose level of 1000 mg/kg of body weight and observed for 24 hours. If mortality was observed in 2 out of 3 animals, then the dose administered was assigned as toxic dose. If mortality was observed in 1 animal, then the same dose was repeated again to confirm the toxic dose. If mortality was not observed, the procedure was repeated for further higher dose such as 2000 mg/kg and 3000 mg/kg of body weight.

Experimental Design

Hepatoprotective activity:

To evaluate Hepatoprotective activity of *Phyllanthus virgatus* in experimental animalsi) Healthy albino Wistar rats of either sex weighing between 200-220gm were selected.ii) Animals were divided randomly into five groups each consisting of six animals

Methodology

CCl_4 induced hepatoxicity

In the CCl_4 induced liver toxicity model, CCl_4 (1 ml/kg i.p.) was administered daily for 7 days to all animals except group 1 [11]. Phyllanthus virgatus whole plant extract low dose (100mg/kg) and high dose (200mg/kg)administrated to treatment groups once daily for 7 days.

Group-1: normal control administered with normal saline (1ml/kg, p.o)

Group-2: toxin control - CCl_4 in Olive Oil (1:1 ratio) (1ml/kg, i.p)

Group-3: standard - drug silymarin (50mg/kg, p.o) and CCl_4 (1ml/kg, i.p)

Group-4: CCl_4 induced hepatotoxic rats treated with methanolic extract of *Phyllanthus virgatus* at a dose of 100mg/kg, p.o. once daily for 7 days.

Group-5: CCl_4 induced hepatotoxic rats treated with methanolic extract of *Phyllanthus virgatus* at a dose of 200mg/kg, p.o. once daily for 7 days.

The animals were sacrificed 24 h after last treatment under light anesthetic ether. Blood from each rat was withdrawn by retro orbital plexus under ether anesthesia for biochemical investigation i. e. SGOT, SGPT, total bilirubin and total protein estimation. Blood was further allowed to coagulate at 37^0C for 30 min and the serum was separated by centrifugation at4000 rpm for 15 min.

Statistical analysis

Results were expressed as Mean±S.E.M. Statistical analysis was performed by one way ANOVA followed by Tukey test with the help of Graph Pad Prism 5.0 software.

Result

Evaluation of administration of Methanolic extract of Phyllanthus virgatus at different doses is given in tabular form:

Table 1: Effect of administration of Methanolic extract of Phyllanthus virgatusextract (100 mg/kg, p.o) + Phyllanthus virgatus whole plant extract (200 mg/kg, p.o) + Silymarin (50 mg/kg, p.o), for 7days on serum (AST or SGOT) levels level in ccl4 induced hepatotoxicity.

GROUP	TREATMENT	MEAN ± SEM
Group 1	Vehicle control	43.48±0.09
Group 2	CCl_4 control	102.73±0.21***
Group 3	Standard(50 mg/kg, p.o)	48.25±0.14***
Group4	Met. ext. of*P. virgatus*(100mg/kg, p.o)	68.19±0.10***
Group 5	Met. ext. of*P. virgatus*(200 mg/kg, p.o)	62.38±0.11**

Values are expressed as Mean±S.E.M; n=6, *P<0.05, **P<0.01, ***P<0.001 considered for significance, (ANOVA followed by tukey test)

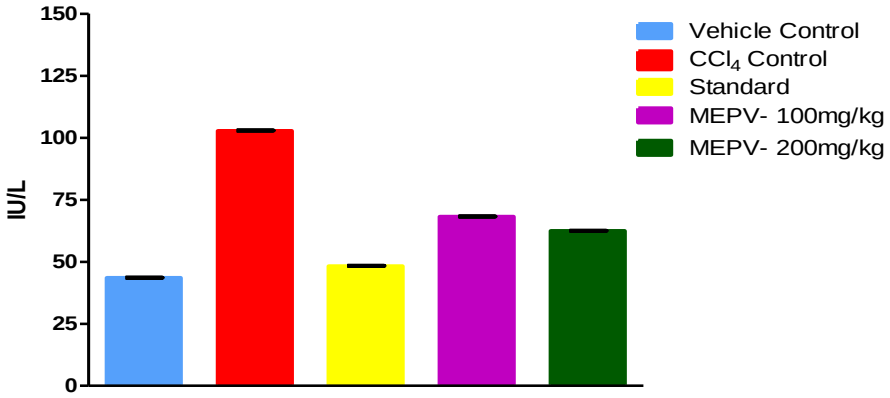

Fig. 1: Effect of *P.virgatus* on SGOT levels in CCl_4 induced hepatoxicity

Table 2: Effect of administration of *Methanolic extract of Phyllanthus virgatus*extract (100 mg/kg, p.o) + *Phyllanthus virgatus whole plant* extract (200 mg/kg, p.o) + Silymarin (50 mg/kg, p.o), for 7days on serum (ALT or SGPT) levels level in CCl_4 induced hepatotoxicity.

GROUP	TREATMENT	MEAN ± SEM
Group 1	Vehicle control	72.79±0.215
Group 2	CCl_4 control	385.75±0.70***
Group 3	Standard(50 mg/kg, p.o)	57.24±0.23***
Group 4	Met. ext. of*P. virgatus*(100mg/kg, p.o)	60.51±0.22***
Group 5	Met. ext. of*P. virgatus*(200 mg/kg, p.o)	59.20±0.27***

Values are expressed as mean±S.E.M; n=6, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ considered for significance, (ANOVA followed by tukey test)

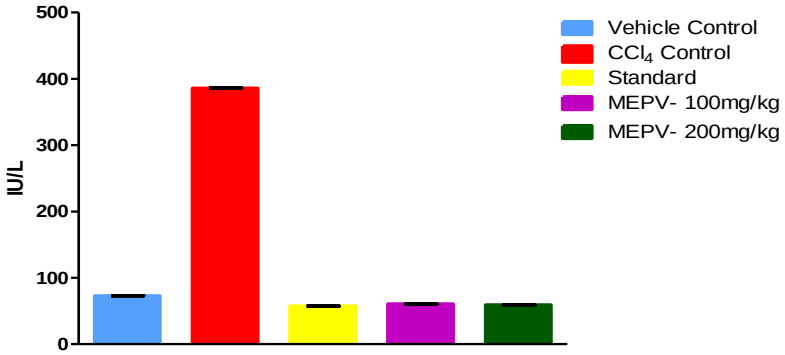

Fig. 2: Effect of *P.virgatus* on SGPT levels in CCl_4 induced hepatoxicity

Table 3:Effect of administration of *Methanolic extract of Phyllanthus virgatus* extract (100 mg/kg, p.o) + *Phyllanthus virgatus whole plant* extract (200 mg/kg, p.o) + Silymarin (50 mg/kg, p.o), for 7days on serum Total bilirubin level in CCl_4 induced hepatotoxicity

GROUP	TREATMENT	MEAN ± SEM
Group 1	Vehicle control	0.26±0.04
Group 2	CCl_4 control	2.05±0.17***
Group 3	Standard(50 mg/kg, p.o)	0.30±0.009*
Group 4	Met. ext. of*P. virgatus*(100mg/kg, p.o)	0.35±0.07***
Group 5	Met. ext. of*P. virgatus*(200 mg/kg, p.o)	0.29±0.008***

Values are expressed as mean±S.E.M; n=6, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ considered for significance, (ANOVA followed by tukey test)

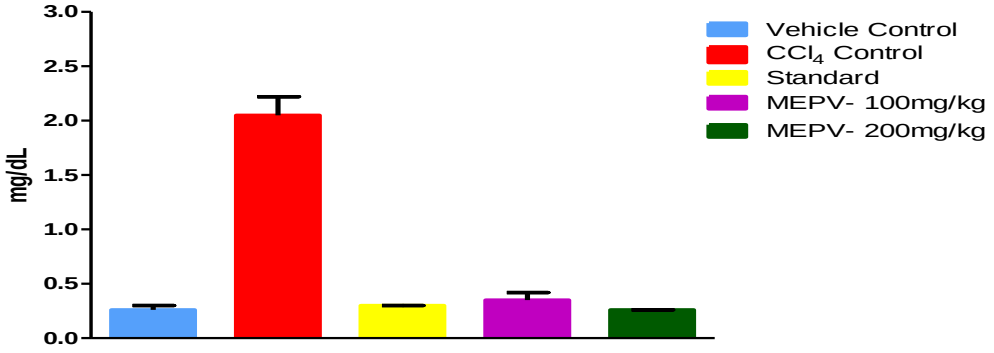

Fig. 4.3: Effect of *P.virgatus* on total bilirubin levels in CCl_4 induced hepatoxicity

Table 4:Effect of administration of *Methanolic extract of Phyllanthus virgatus*extract (100 mg/kg, p.o) + *Phyllanthusvirgatus whole plant* extract (200 mg/kg, p.o) + Silymarin (50 mg/kg, p.o), for 7days on serum Total protein level in CCl_4 induced hepatotoxicity

GROUP	TREATMENT	MEAN ± SEM
Group 1	Vehicle control	7.48±0.87
Group 2	CCl_4 control	2.16±0.01***
Group 3	Standard(50 mg/kg, p.o)	6.80±0.07***
Group 4	Met. ext. of*P. virgatus*(100 mg/kg, p.o)	3.73±0.006***
Group 5	Met. ext. of*P. virgatus*(200 mg/kg, p.o)	6.15±0.02***

Values are expressed as Mean±S.E.M; n=6, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ considered for significance, (ANOVA followed by tukey test)

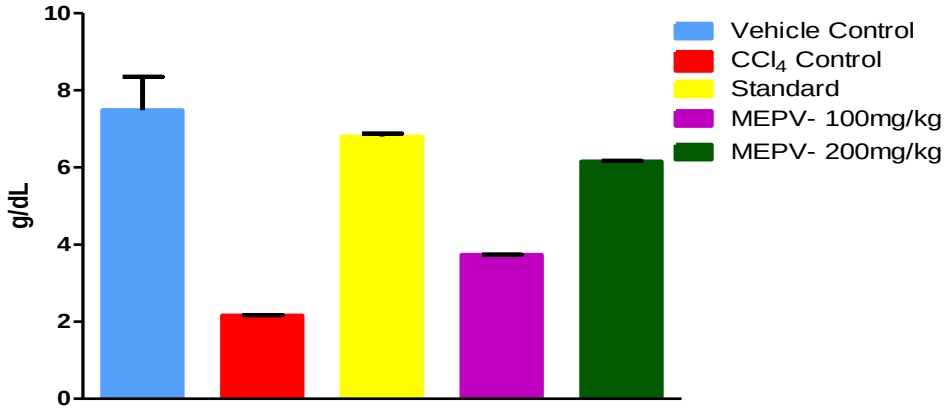

Fig4: Effect of administration of *Methanolic extract of Phyllanthus virgatus* extract (100 mg/kg, p.o) + *Phyllanthusvirgatus whole plant* extract (200 mg/kg, p.o) + Silymarin (50 mg/kg, p.o), for 7days on serum Total protein level in ccl4 induced hepatotoxicity

Table 5: Percentage Recoveryof Carbon tetrachloride

TREATMENT	DOSE	% Reduction of Serum Biomarkers			%Protection of Serum Biomarkers
Methanolic extract of *Phyllanthus virgatus*		SGOT	SGPT	Total Bilirubin	Total Protein
	100 mg/kg, p.o	58	93	94	29
	200 mg/kg, p.o	68	95	96	75
Standard drug Silymarin	50 mg/kg, p.o	91	96	97	87

Discussion

Liver plays a major role in detoxification and excretion of many endogenous and exogenous compounds, any injury or impairment of its function may lead to several implications on one's health. Management of liver diseases is still a challenge to modern medicine [12]. Conventional drugs used in the treatment of liver diseases are often inadequate. It is therefore necessary to search alternative drugs for the treatment of liver diseases and to replace the currently used drugs of doubtful efficacy and safety.

Morbidity and mortality resulting from chronic liver diseases such as hepatitis is a major public health problem worldwide especially in developing countries. The major abnormalities associated with hepatitis are lipidemia, per oxidation and loss of plasma membrane integrity. Search for new drugs for limiting hepatic injury has been of interest recently with the better understanding of the processes involved in hepatitis fuelled by the urgent need for the clinical development of safe and non-toxic cytoprotective agents [13].

The use of rats as experimental animals for Hepatoprotective activity is mainly because of the structural homology of rat CYP 450 enzymes with that of humans. Female rats are less susceptible to CCl_4-induced liver damage, especially hydroxyproline accumulation [14].

Carbon tetrachloride is a routinely used hepatotoxin for experimental study of liver diseases Administration of CCl_4 causes acute liver damage that mimics natural causes. It mediates changes in liver function that ultimately leads to destruction of hepatocellular membrane. CYP 450 activates CCl_4 to form various free radicals (trichloromethyl, Cl_3C-CCl_3 (hexachloroethane), $COCl_2$ (phosgene), etc.) which are involved in the pathogenesis of liver damage in chain reactions resulting in per oxidation of lipids, covalent binding of macromolecules, disruption of metabolic mechanisms in mitochondria, decreasing levels of phospholipids, increasing triglyceride levels, inhibition of calcium pumps of microsomes thus leading to liver necrosis [15].

The acute toxicity study revealed the absence of lethality among the tested animals when the extract (p.o) was administered as a single dose (1000mg/kg, 2000mg/kg and above). There were no signs of any gross behavioral changes except for an increase in urination indicating the safe usage of the extract at a dose of 100 mg/kg and 200 mg/kg.

Necrosis or membrane damage releases the enzymes into circulation and hence it can be measured in the serum. The reversal of increased serum enzymes in CCl_4-induced liver damage by the extract may be due to the prevention of the leakage of intracellular enzymes by its membrane stabilizing activity.

Amino transferees contribute a group of enzymes that catalyse the interconversion of amino acids and α-keto acids by the transfer of amino groups. These are liver specific enzymes and are considered to be very sensitive and reliable indices for necessary hepatotoxic as well as hepatoprotective or curative effect of various compounds Aminotransferase include aspartate aminotransferase (AST or SGOT) and alanine aminotransferase (ALT or SGPT). These enzymes are normally predominantly contained within liver cells and to a lesser degree in the muscle cells. If the liver is injured or damaged, the liver cells spill these enzymes into the blood, raising the AST and ALT enzyme blood levels and signaling liver disease.

Both AST and ALT levels increase due to toxic compounds that affect the integrity of liver cells. Decreased levels of transaminases indicate stabilization of plasma membrane and protection of hepatocytes against damage caused by hepatotoxic. Both the doses of methanolic extract of *Phyllanthusvirgatus* could significantly lower the elevated amino transferase levels when compared to both carbon tetrachloride group. This is in agreement with the commonly accepted view that serum levels of transaminases return to normal with the healing of hepatic parenchyma and the regeneration of hepatocytes

Bilirubin is produced by the normal breakdown of pigment-containing proteins, especially hemoglobin from senescent red blood cells and myoglobin from muscle breakdown. Bilirubin released from such sources, tightly albumin bound, is delivered to the liver, where it is efficiently extracted and conjugated by hepatic glucuronidation and sulfation. Conjugated bilirubin is rapidly excreted into bile and removed from the body through the gut. An elevated level of conjugated serum bilirubin implies liver disease. Normally, 90% or more of measured serum bilirubin is unconjugated (indirect-reacting). Elevations of the unconjugated bilirubin level when the conjugated bilirubin level remains normal may also indicate an increased load of bilirubin caused by hemolysis [16].

Measurement of total bilirubin includes both unconjugated and conjugated bilirubin. Higher than normal levels of direct or indirect bilirubin may indicate different types of liver problems There was a remarkable reduction in the bilirubin levelsby both the doses of methanolic extract of *Phyllanthusvirgatus* when comparedto both carbon tetrachloride group, implying itspotential as Hepatoprotective agent.

The liver was also known to play a significant role in the serum protein synthesis, being the source of plasma albumin, fibrinogen and also the other important components like α and β-globulin. The liver is also concerned with the synthesis of γ- globulin. The serum albumin level is low in hepatic diseases. The metabolic biotransformation of amino acids in liver by synthesis, transamination, etc., may be impaired due to the escape of both non-proteins and protein nitrogenous substances from injured cells as mediated by raise in the serum enzyme levels of ALP, AST and ALT . The reduction in the total protein (TP) is attributed to the initial damage produced and localized in the endoplasmic reticulum which results in the loss of CYP 450 leading to its functional failure with a decrease in protein synthesis and accumulation of triglycerides leading to fatty liver [16]. Both the doses of methanolic extract of *Phyllanthusvirgatus* considerably enhanced the synthesis of total protein which may be by accelerating the regeneration process and protecting the liver cells. The increased levels of total protein in serum are indicative of the hepatoprotective activity.

CONCLUSION

Methanolic extract of Phyllantus *virgatus* was selected for the study. The whole plant was extracted with 95% methanol using a soxhlet apparatus.Carbon tetrachloride was selected as toxicants as they are most widely used and responsible for about 30-50% of hepatic disorders.To estimation of serum biochemicalparameters results demonstrated that both the doses of the plant extract of Phyllanthus virgatus (i.e. 100 mg/kg, p.o. and 200 mg/kg, p.o) prevented CCl_4 induced (1ml/kg) hepatotoxicity in rats. It produced dose dependent effects.The methanolic extract of *P.virgatus* decreased the elevated SGOT, SGPT and total bilirubin levels. At a dose of 200mg/kg significant percent reduction was observed.Methanolic extract of *P.virgatus* increased the reduced total protein levels. At a dose of 200mg/kg significant percent protection was observed.

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